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Hemorrhagic Syndrome in Chicks Fed Normal Rations Supplemented With Sulfaquinoxaline.* (21696)

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(Introduced by J. B. Brown.)

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Several groups of investigators (Griminger *et al.*(1,2), Hare *et al.*(3), and Sweet *et al.* (4)), have shown that hemorrhages and prolonged blood clotting time can be produced in battery raised chicks by feeding corn-soybean oil meal rations low in vit. K. These investigators also found that the feeding of vit. K or adequate alfalfa would result in normal clotting time and eliminate the hemorrhagic condition. More recently, Morrison *et al.*(5) and Shelton *et al.*(6) reported that the feeding of high levels of sulfaquinoxaline accentuated vit. K deficiency in chicks fed a corn-soy ration. The use of alfalfa or supplementary vit. K counteracted the effects of high levels of sulfaquinoxaline.

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The purpose of this paper is to report the production of a hemorrhagic syndrome in battery raised chicks fed practical chick starter or broiler rations adequate in alfalfa and vit. K. Moore *et al.*(7), observed an outbreak of a hemorrhagic syndrome at the Ohio Agricultural Experiment Station in chicks fed the 1954 Ohio Chick Starter(8). The chicks had been treated with 0.06-0.1% sulfaquinoxaline in the feed prior to the outbreak of hemorrhagic disease. When the same feed, containing 0.1% sulfaquinoxaline, was fed to ten 7 week old Leghorn cockerels housed in a battery, 2 of the birds developed very pale combs and hemorrhages in the musculature of the thighs and shanks. An autopsy revealed extensive internal hemorrhages. These observations prompted the experiments reported herein.

Procedure. Sexed White Plymouth Rock

chicks were used in the 2 experiments conducted. The chicks were housed in batteries and were distributed into groups containing equal numbers of males and females. The basal ration used in Exp. 1 was the 1954 Ohio Chick Starter minus an antibiotic. The percentage composition of this ration is as follows: yellow corn 40, wheat middlings 15, wheat bran 7, solvent extracted soybean oil meal 22.5, meat scrap (50% protein) 5, dehydrated alfalfa meal (17% protein) 5, fish meal 3, defluorinated rock phosphate 1, limestone 1, iodized salt 0.5, manganese sulfate 0.025, dl methionine 0.025. Vitamins added per pound of feed: vit. A 1,000 I. U., vit. D_3 225 I|C.U., riboflavin 1.6 mg, calcium pantothenate 1 mg, niacin 6 mg, choline chloride 125 mg. In Exp. 2 the Ohio Chick Starter ration mentioned above was also used but was supplemented with 2 mg of procaine penicillin G and 5 mg menadione per pound. Some groups were fed a high energy broiler ration of the following percentage composition: yellow corn 51.3, wheat middlings 5, solvent extracted soybean oil meal 30, meat scrap (50% protein) 5, dehydrated alfalfa meal (17% protein) 3, fish meal 3, defluorinated rock phosphate 1.2, limestone 1, iodized salt 0.5, manganese sulfate 0.025, dl methionine 0.025. Vitamins added per pound of feed: vit. A 1500 I.U., vit. D_3-300 I.C.U., menadione 5 mg, riboflavin 2 mg, calcium pantothenate 2 mg, choline chloride 180 mg, niacin 10 mg, vit. B_{12} 6 mg. Procaine penicillin G was added at a level of 2 mg per pound. All chicks used in Exp. 2 were weighed at 3 weeks and distributed into uniform groups based on body weight before the drug treatments were started. Blood was obtained by etherizing the chicks, opening the thoracic cavity, and sampling the blood by heart puncture. Wintrobe tubes were used for the hematocrit determinations. Whole blood clotting time was measured using the method of Lee and White (9), and prothrombin time using the method of Quick(10). Post mortem examinations were made of all chicks that died during the experiment. In addition, representative birds were killed and examined for signs of hemorrhage. The "t" test of Snedecor(11) was used in the statistical analysis of the data. The following formula for comparing groups with different numbers of individuals was used:

$$t = \frac{\overline{x}}{\sqrt{\frac{n_1 n_2 (n_1 + n_2 - 2)}{(n_1 + n_2)\, Sx^2}}}.$$

Results. The experimental plan and results of Exp. 1 are presented in Table I.

In the absence of penicillin, the addition of 0.1% sulfaquinoxaline to the basal ration at 3 weeks had no effect on hematocrit at 7 weeks of age; (treatment 2) however, 25% of the chicks showed external signs of hemorrhages at 4 weeks of age. The hemorrhages occurred primarily under the skin of the shanks. The external hemorrhages in this group disappeared when the birds were 6 weeks old. In the presence of penicillin (treatment 7) the addition of 0.1% sulfaquinoxaline at 3 weeks of age resulted in a statistically significent decrease in hematocrit as well as a high incidence of external hemorrhages. These data indicate that dietary penicillin tended to increase the toxicity of sulfaquinoxaline.

The continuous feeding of sulfaquinoxaline at levels of 0.1 and 0.15% (treatments 4 and 5) did not affect the hematocrit at 7 weeks and did not result in any external signs of hemorrhage. This is in agreement with the report of Merck and Co.(12). Several of the birds which died in these groups, however, showed hemorrhagic lesions on post mortem examination. The feeding of 0.1% sulfaquinoxaline starting at 2 weeks of age also had little effect on hematocrit at 7 weeks or on the production of external signs of hemorrhage.

Fig. 1 shows the hematocrit of chicks fed 0.1% sulfaquinoxaline at different ages. Feeding the drug at 3, 4 or 5 weeks of age resulted in decreased hematocrit when measured at 7 weeks of age while chicks fed the drug at day-old or 2 weeks showed essentially normal hematocrits. This may be interpreted in 2 ways; either the drug had no effect on the hematocrit of birds 0-2 weeks of age, or, birds fed the drug at 0-2 weeks were able to overcome the effect of the drug by the 7th week when the hematocrit determinations were made. The feeding of 0.05% sulfaquinoxaline starting at 5 weeks had no effect on hema-

TABLE I. Effects of Feeding Sulfaquinoxaline on Hematocrit, Clotting, Incidence of Visible Hemorrhage and Mortality.

Treatment No.	No. of chicks	Ration	Level of sulfa, %	Age at which sulfa added to ration	Measured at 7 wk: Hematocrit, mean ± S.D. and range	Measured at 7 wk: Avg clotting time, sec	Measured at 7 wk: Avg prothrombin time, sec	Incidence of externally visible hemorrhages, %	Total mortality 8 wk, %	Mortality showing internal hemorrhages, %
1	45	Chick starter	.0	—	24.9 ± 1.6 (22.5-28.6) (16)†			.0	6.6	.0
2	40	*Idem*	.1	3 wk	25.6 ± 4.3 (16.3-32.2) (21)			25.0	10.0	2.5
3	40	as 1 + 4 g proc. penicillin/ton.	.0	—	26.0 ± 4.1 (19.8-32.1) (24)	189 (7)	181 (7)	.0	2.5	.0
4	40	*Idem*	.1	1 day	26.7 ± 3.8 (21.8-36.7) (18)			.0	7.5	5.0
5	48	"	.15	"	26.5 ± 3.1 (22.1-34.1) (16)			.0	8.3	2.0
6	40	"	.1	2 wk	25.4 ± 4.4 (18.3-33.1) (15)			2.5	7.5	7.5
7	40	"	.1	3 "	22.7 ± 4.9‡(10.0-28.7) (12)			32.5	12.5	7.5
8	40	"	.1	4 "	23.0 ± 6.1 (9.44-30.4) (8)			5.0	15.0	5.0
9	40	"	.05	5 "	28.5 ± 6.3 (9.70-35.2) (15)			5.0	5.0	2.5
10	40	"	.1	5 "	22.4 ± 5.2‡(9.16-31.1) (20)	140 (13)	173 (12)	2.5	7.5	5.0
11	40	as 3+ vit. mixture*	.0	—	28.2 ± 4.0 (21.1-34.1) (21)	229 (7)	160 (7)	.0	.0	.0
12	40	*Idem*	.1	3 wk	27.3 ± 4.3 (20.1-34.3) (14)			12.5	15.0	5.0
13	40	"	.1	5 "	21.4 ± 6.5§(5.6-31.7) (33)	352 (12)	196 (11)	22.5	5.0	5.0
Pooled data		No sulfaquinoxaline (125)†			26.4			.0	3.0	.0
		Plus sulfaquinoxaline (408)			25.0 (significant at 5% level)			10.8	9.3	4.7

* Vitamins added/lb of feed: Folic acid, 2 mg; ascorbic acid, 20 mg; para amino benzoic acid, 50 mg; rutin, 20 mg; alpha-tocopheryl acetate, 17 mg; menadione, 5 mg; vit. A, 2000 I.U.; biotin, 0.2 mg. † No. of chicks in parenthesis. ‡ Significant at 5% level when compared with treatment 3. § Significant at 1% level when compared with treatment 11 or 3.

TABLE II. Effects of Feeding Sulfaquinoxaline and Iodinated Casein plus Sulfaquinoxaline on Incidence of Hemorrhages.

No. of chicks	Ration	Level of sulfaquinoxaline, %	Age at which sulfaquinoxaline fed, wk	Hematocrit at 5 wk, mean ± S.D. and range	Avg clotting time at 5 wk, sec	Total mortality 6 wk, %	Mortality showing hemorrhages, %	Incidence of hemorrhage in birds killed at 5 wk, %
32	CS‡	.0	—	27.4 ± 3.4 (21.3-32.6) (13)†	158 (15)†	.0	.0	.0 (16)†
32	CS‡	.1	3	26.6 ± 3.8 (18.1-31.3) (15)	340 (13)*	3.1	3.1	23.5 (17)
34	CS‡ + .5 g iodinated casein/lb starting at 3 wk	.1	3	24.1 ± 6.6 (8.7-35.5) (13)	443 (13)	14.7	11.8	31.2 (16)
33	Broiler ration	.0	—	—	—	0	0	—
34	*Idem*	.1	3	—	—	11.8	11.8	—
33	CS‡ (chicks on litter at 3 wk)	.0	—	—	—	9.1	0	—
33	*Idem*	.1	4	—	—	24.2	6.1	—

* Significant at 2% level. † No. of chicks in parenthesis. ‡ Containing 2 mg procaine penicillin G and 5 mg menadione/lb.
CS = Chick starter.

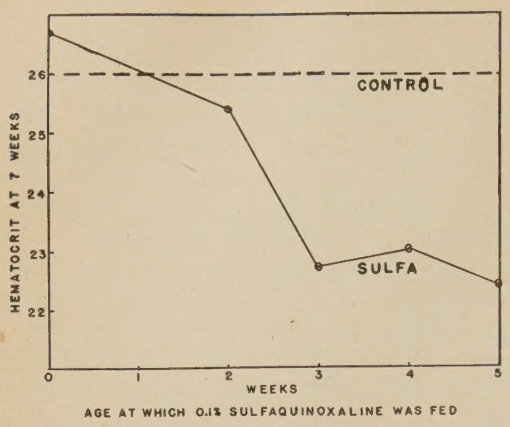

FIG. 1. Effect of feeding sulfaquinoxaline at different ages on hematocrit.

tocrit, but did result in some cases of hemorrhage.

Supplementation of the basal ration with a vitamin mixture, the composition of which is shown under Table I, resulted in an increased hematocrit. The vitamin mixture supplied 5 mg of menadione per pound of feed. The difference in hematocrit between treatments 1 and 11 was highly significant. The addition of 0.1% sulfaquinoxaline at 3 weeks of age, in the presence of the added vitamin supplement, had no significant effect on hematocrit. When sulfaquinoxaline was added at 5 weeks of age (treatment 13) there was a highly significant decrease in hematocrit and an appreciable number of chicks showed external symptoms of hemorrhages. Thus the additional vitamin mixture appeared to partially protect the chicks against the toxic effects of sulfaquinoxaline administered at 3 weeks but had no effect in chicks administered the drug at 5 weeks.

The variability in hematocrit data may be explained by the observation that the chicks appeared pale and anemic soon after sulfaquinoxaline treatment was started. After a period of stress, the majority of birds were apparently able to adapt. This is in accordance with the field observations of hemorrhagic disease reported by Gray *et al.*(13) in which recovery in many cases occurred spontaneously. Similar cases of spontaneous recovery in field cases of hemorrhagic syndrome have been observed by the authors. Another cause of variability in the hematocrit data was the fact that some chicks were resistant to the toxic effects of sulfaquinoxaline and therefore showed normal hematocrits while other chicks in the same pen showed hemorrhages. The chicks with severe hemorrhages had very low hematocrits, some as low as 5.6. None of the control chicks showed hematocrits lower than 19.8. Gray *et al.*(13) reported variable hematocrits in field cases of hemorrhagic disease. In the birds tested by these investigators the hematocrits ranged from 5 to 31 with an average of 21.6.

When the mean hematocrits of all the groups receiving sulfaquinoxaline were pooled and compared with the means of the control groups a small difference was observed. These pooled means are shown at the bottom of Table I. When the difference between the pooled means was analyzed statistically using the "t" test of Snedecor(11) it was found to be significant at the 5% level. When the individual hematocrits were pooled rather than the means the pooled figures were 26.5 for the controls and 24.7 for the chicks receiving sulfaquinoxaline. The difference between these weighted means was significant at the 1% level. A total of 233 hematocrit determinations were used in these pooled analyses.

Post mortem examinations of chicks which died or were killed during the course of the experiment revealed the presence of hemorrhages closely resembling those encountered in field cases of hemorrhagic syndrome as reported by Gray *et al.*(13) and observed at our laboratory. The incidence of these hemorrhages in birds which died during the experiment is shown in the last column of Table I. Gross lesions at autopsy included pale yellowish flesh, thin watery blood, edema and congestion of the lungs in some birds, yellow fatty bone marrow and hemorrhages. Petechial hemorrhages were seen in the skin. Feather follicles showed some hemorrhage. Breast, thigh and leg muscles contained linear, ecchymotic and diffuse hemorrhages which were both superficial and deep. Linear hemorrhage was present in the heart. Livers and intestines contained countless numbers of petechial and ecchymotic hemorrhages in many birds. No coccidia were found on re-

FIG. 2. Hemorrhages in 7-wk-old chick fed 0.1% sulfaquinoxaline 2 weeks.

peated intestinal scrapings. Two birds appeared to have brain hemorrhages. Fig. 2 is an example of a chick with hemorrhages due to feeding 0.1% sulfaquinoxaline.

The incidence of hemorrhages in both live and dead birds was variable, however, an examination of the pooled data presented at the bottom of Table I shows that none of the control birds had any hemorrhages while the groups treated with sulfaquinoxaline had hemorrhages.

Clotting time and prothrombin time were determined on some chicks. In the absence of the additional vitamin mixture clotting time and prothrombin time were normal in the presence of sulfaquinoxaline despite the fact that a marked anemia was evident in many of the birds tested. In the groups receiving sulfaquinoxaline plus the added vitamin mixture sulfaquinoxaline increased the blood clotting time; however, due to the wide variability between individual birds these differences were not statistically significant.

The results of Exp. 2 (Table II) confirm the observations made in Exp. 1. A higher incidence of mortality due to hemorrhage was observed in the chicks fed the broiler ration as compared with the chick starter. This may have been due to the more rapid growth obtained with the broiler ration as shown in Table III. Since "stress factors" have been reported to be involved in field cases of hemorrhagic disease(12) iodinated casein was fed to some groups in addition to sulfaquinoxaline. The feeding of iodinated casein increased both the incidence and severity of hemorrhages. In other experiments not reported in this paper the feeding of iodinated casein alone did not result in the production of hemorrhages.

Clotting time was increased by the feeding of sulfaquinoxaline despite the fact that the ration contained 5% alfalfa and 5 mg menadione per pound. The failure to prevent prolonged blood clotting time with the relatively high level of menadione and alfalfa may mean that sulfaquinoxaline is interfering with blood clotting through some mechanism not involving prothrombin. The variability in clotting and prothrombin time is shown in Table IV. The growth data presented in Table III demonstrate that the feeding of sulfaquinoxaline at 3 weeks of age resulted in a marked growth

TABLE III. Effect of Feeding Sulfaquinoxaline and Iodinated Casein plus Sulfaquinoxaline on Chick Growth (Exp. 2).

No. of chicks	Ration	Sulfaquinoxaline level, %	Age at which sulfaquinoxaline fed, wk	Avg 5 wk wt (both sexes), g	Avg of duplicate pens, g
16, 16	CS*	—	—	467, 477	472
16, 15	CS*	.1	3	422, 423	423
17, 16	CS* + .5 g iodinated casein/lb†	.1	3	397, 410	404
16, 16	BR‡	—	—	532, 527	530
17, 17	BR‡	.1	3	444, 450	447
17, 16	CS* (chicks put on litter at 3 wk)	—	—	467, 469	468
16, 17	*Idem*	.1	4	466, 470	468

* CS = Chick starter. Containing 5 mg menadione/lb and 2 mg procaine penicillin G/lb.
† Iodinated casein started at 3 wk.
‡ BR = Broiler ration.

TABLE IV. Variability in Clotting Time and Prothrombin Time.

Exp. No.	Ration	Level of sulfa, %	Age at which sulfa added to ration, wk	Clotting time (sec) Mean ± S.D. and range	Prothrombin time (sec) Mean ± S.D. and range
1	CS + 4 g proc. penicillin/ton.	.0	—	189 ± 119 (7)* (51- 420)	181 ± 93 (7)* (113-364)
	Idem	.1	5	140 ± 87 (13) (54- 232)	173 ± 54 (12) (79-261)
	" + vit. mixture†	.0	—	229 ± 179 (7) (45- 580)	160 ± 68 (7) (95-290)
	Idem	.1	5	352 ± 359 (12) (30-1234)	196 ± 79 (11) (94-326)
2	CS‡	.0	—	158 ± 151 (15) (30- 600)	—
	Idem	.1	3	340 ± 210§ (13) (85- 600)	—
	" + .5 g iodinated casein/lb starting at 3 wk	.1	3	443 ± 678 (13) (173-1762)	—

* No. of chicks in parenthesis. † See bottom of Table I for composition of vitamin mixture.
‡ Containing 2 mg procaine penicillin G and 5 mg menadione/lb. § Significant at 2% level.

depression by the 5th week. On the other hand, the feeding of the drug starting at 4 weeks had no effect on the 5 week weights. These data along with other observations indicate that, under these conditions, the toxic effect of sulfaquinoxaline in most cases, is evident after 10 to 14 days of treatment.

The chicks which were put on litter at 3 weeks had higher mortality than similar chicks housed in batteries. Some of the mortality was due to coccidiosis even though a high level of sulfaquinoxaline was fed. Coccidiosis was noted in the treated chicks as well as in the controls. Kendall and McCullough(14), using sulfamezathine, also reported that an excessively high concentration of the drug may fail to control coccidiosis.

While this paper was being prepared, two reports were presented at the World's Poultry Congress dealing with the toxic effects of sulfaquinoxaline in chicks. These reports, by Davies(15) and Goldhaft and Wernicoff(16) show that sulfaquinoxaline will produce hemorrhages in chicks. The work reported in this paper is in agreement with these reports and furnishes additional information regarding the effect of diet and stress factors on the hemorrhagic syndrome induced by sulfaquinoxaline.

Summary. 1. Feeding of 0.1% sulfaquinoxaline in the presence of 3-5% alfalfa and 5 mg of menadione per lb. of feed resulted in occurrence of a hemorrhagic syndrome in chicks. Hemorrhages closely resembled those encountered in field cases of hemorrhagic disease and, in many cases were accompanied by reduced hematocrits. In some groups blood clotting time was increased by feeding sulfaquinoxaline. A higher incidence of hemorrhages was observed in chicks fed sulfaquinoxaline in broiler rations than in chicks fed starter rations containing this drug. Feeding of iodinated casein as a stress factor increased the toxic effect of sulfaquinoxaline. The toxic effect of sulfaquinoxaline was slight in chicks fed the drug starting at day-old or at 2 weeks of age but was more pronounced in chicks fed the drug at 3, 4 or 5 weeks of age. Dietary penicillin appeared to increase the toxicity of sulfaquinoxaline. 2. The adverse effects of sulfaquinoxaline in these experiments are not to be interpreted to mean that sulfaquinoxaline and similar substances cannot be used as satisfactory coccidiostats under certain conditions.

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Effect of Hypophysectomy on Liver Nucleoproteins.* (21697)

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Di Stefano, *et al.*(1) have shown that hypophysectomy results in a decrease in ribonucleic acid (RNA) and protein of liver cells. Microspectrophotometric measurements made indicated that there was no change in the deoxyribonucleic acid (DNA) content of spherical nuclei having a diameter of about 7 μ. Chemical analysis, however, revealed an increase in DNA per unit weight of liver, which was explained as being due to an increase in the cellularity of the tissue. Swift (2) suggested that the data presented could be explained, at least in part, by a change in polyploid frequencies. Leuchtenburger, *et al.*, (3) recently has reported that dwarf mice with congenital absence of the hypophysis have no tetraploid or octaploid liver nuclei. It seemed, therefore, of some interest to reinvestigate the effect of hypophysectomy on liver nucleoproteins. The modifications in DNA were evaluated both by determining the amount of nucleic acid per average nucleus and by microspectrophotometric methods employing the Feulgen stain.

Methods. Chemical Analyses: Male rats of the Sprague-Dawley strain were obtained from the Endocrine Laboratories, Madison, Wis. All animals were maintained on a diet of canned dog food and allowed water *ad libitum*. Four days post-operatively the animals were divided into 3 groups as indicated in Table I. One group of intact controls was given daily intramuscular (IM) injections of 0.1 cc of 0.85% saline for 7 days. One group of hypophysectomized rats received 0.5 mg of growth hormone† daily for 7 days. The remaining group of hypophysectomized rats received the same saline injections as the intact controls. All animals were weighed daily. On the day following the last injection, the rats were sacrificed and the organs selected for study were rapidly removed and cooled to 0°C. A 10% saline homogenate was prepared. Nucleic acids were extracted and determined by the method of Schneider(4). Nitrogen was estimated by the micro-Kjedahl method of Ma and Zuazaga(5). Nuclei counts were made according to a method previously described by us(6). The DNA per average nucleus was calculated from the number of nuclei and amount of DNA in a unit volume of the tissue homogenate. *Microspectrophotometric methods*: The rats employed had been hypophysectomized for 21 days. Tis-

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† The growth hormone, Lot No. R-5270943, was generously supplied through the courtesy of Mr. Irby Bunding, Armour Laboratories, Chicago, Ill.

TABLE I. Effect of Growth Hormone on the Nucleoproteins of the Liver of Normal Rats.

	Hypox (6)	Control (12)	Hypox + growth hormone (8)
Body wt, g	141.0 ± 1.72*	226.0 ± 4.23	183.38 ± 2.9
		P <.01	P <.01
Liver wt, g	4.74 ± .137	8.5 ± .399	6.19 ± .15
		P <.01	P <.01
% dry wt of liver	30.09 ± .423	29.56 ± .293	28.24 ± .21
		P <.4	P <.01
Liver N, mg/100 mg	3.55 ± .073	3.41 ± .045	3.28 ± .04
		P <.2	P <.05
Nuclei/g × 10^8	5.03 ± .169	3.83 ± .063	4.18 ± .11
		P <.01	P <.02
DNA phosphorus, mg/100 g	49.37 ± 1.16	35.14 ± .997	42.15 ± 1.4
		P <.01	P <.01
DNA/nucleus (mg × 10^{-9})	10.17 ± .189	9.05 ± .135	10.2 ± .23
		P <.01	P <.01
RNA phosphorus, mg/100 g	89.30 ± .687	106.38 ± 1.41	107.4 ± 2.4
		P <.01	P <.8

* All figures represent a mean ± S. E. S. E. computed by: S. D. $= \sqrt{\frac{\Sigma x^2 - (Mx\,\Sigma x)}{n-1}}$; S. E. $= \frac{\text{S. D.}}{\sqrt{n}}$.

P = Probability from Fischer's Table "t."

sues were prepared for microspectrophotometric analysis as previously described(1). The livers of 3 control and 3 hypophysectomized animals were studied. Approximately 150 cells were selected in each tissue for DNA determination. The sections were cut at 10 μ because in hypophysectomized animals, measurements could not be made on thicker sections due to extensive over-lapping of nuclei. A Beckman DU spectrophotometer was used as a light source, and all readings were made employing a wave-length of 560 mμ. The extinctions were determined on a nuclear plug and were then converted to units of DNA

TABLE II. Class Distribution of Nuclei in Liver from Normal and Hypophysectomized Rats.

Ploidy class	Animal	Animal No.	No. of nuclei	% in class	DNA units, mean ± S.E.
Class I (diploid)	Control	1	31	19.7	8.406 ± .197
4.1-12.1 DNA units		2	33	21.2	8.858 ± .214
		3	35	22.4	8.389 ± .265
	Hypox	1	43	27.4	8.702 ± .250
		2	45	28.8	8.982 ± .179
		3	43	27.4	8.490 ± .211
Class II (tetraploid)	Control	1	113	71.9	16.950 ± .185
12.6-22.2 DNA units		2	111	71.2	16.745 ± .200
		3	108	69.2	16.582 ± .194
	Hypox	1	77	49.3	17.162 ± .752
		2	80	51.3	16.871 ± .646
		3	77	49.3	16.699 ± .388
Class III (octaploid)	Control	1	13	8.4	28.523 ± .758
22.7-39.5 DNA units		2	12	7.6	28.883 ± .354
		3	13	8.4	29.023 ± .828
	Hypox	1	36	23.4	28.840 ± .748
		2	31	19.9	27.797 ± .623
		3	36	23.4	28.503 ± .650

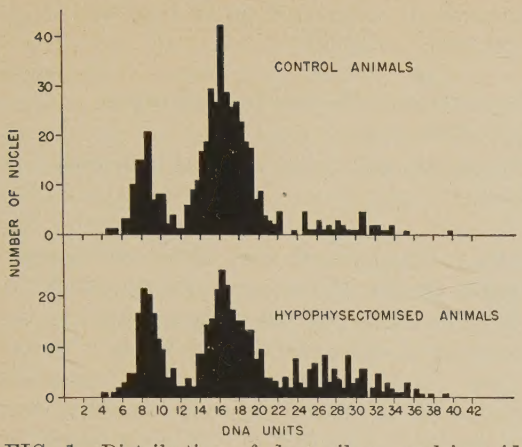

FIG. 1. Distribution of deoxyribose nucleic acid in nuclei from normal and hypophysectomized rats.

using the formula of Swift for nuclear volumes(7). Nuclei were selected for analysis using two criteria: 1) that they were whole nuclei of liver parenchymal cells, and 2) that they were spherical.

Results. In Table I the data obtained by chemical analysis are shown. The data confirm the earlier reports of Di Stefano, *et al.*, (1); however, in hypophysectomized animals it was observed that there was a significant increase in DNA/average nucleus, an increase which did not rapidly return to normal when growth hormone was administered. It is apparent that changes in tissue constituents of liver can not be satisfactorily estimated by assuming that the average DNA per cell is constant. From Table II and Fig. 1, the reason for the changes in DNA following hypophysectomy is shown. Because the sections studied were cut at 10μ, the percentage of octaploid nuclei is lower than would have been obtained had the sections been thicker. It is quite evident, nonetheless, that in hypophysectomy there is an increase in diploid and octaploid nuclei accompanied by a decrease in tetraploid nuclei. Also, an 8% increase in DNA is observed in the nuclei of the hypophysectomized animals when the total DNA in units is calculated for all cells in each group of 3 animals. This percentage would have been higher if it had been possible to include all octaploid nuclei. These data support the chemical data shown in Table I.

Summary. Earlier investigations on liver nucleoprotein changes following hypophysectomy have been extended showing that the DNA per average nucleus is increased. This is not restored to normal values rapidly, since treatment for one week with growth hormone effects no appreciable reduction. These data obtained by chemical analyses strongly indicated that following hypophysectomy there was an increase in the frequency of nuclei of higher ploidy. This was found to be the case when the nuclei were examined by microspectrophotometric technics. The results suggest that growth hormone may be important in effecting the conversion of diploid to the tetraploid nuclei. However, tetraploid nuclei may be converted to octaploid nuclei in the absence of growth hormone.

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Increased Excretion of Urinary Corticoids by Guinea Pigs Following Administration of Pitressin.* (21698)

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It was previously reported that an increased excretion of urinary corticoids occurred in the rat following the administration of extracts from beef posterior pituitary glands (P.P.E.) and commercial pitressin(1). The presence of a substance in the posterior pituitary which presumably increases ACTH secretion is consistent with the concept that the stimulus for the release of ACTH by the anterior pituitary gland is humoral(2). The hypothetical substance is released by the hypothalamus and is transported to the anterior pituitary by way of the hypophyseal-portal system(3). The works of other investigators have implicated pitressin in the release of ACTH by the anterior pituitary(4,5). The data presented herein are consistent with this concept.

It was observed that an increased excretion of urinary corticoids occurs in the guinea pig following the intraperitoneal injection of pitressin. The anterior pituitary is necessary for the adrenal cortical stimulation by pitressin and this is presumably mediated through ACTH release. Furthermore the activation of ACTH release was not related to the production of systemic hypertension, hyponatremia, systemic anoxia or adrenalin release.

Procedure. The guinea pig was chosen because it responds to ACTH administration with large increases in corticoid excretion as measured by the urinary neutral reducing lipids (NRL)(6). The observations of Burstein were confirmed and it was also observed that the guinea pig responded to P.P.E. with large increases in NRL excretion. *Urine collections* were carried out according to the method of Burstein except that 15 cc of a solution of saturated ammonium sulphate was introduced into each collection bottle instead of chloroform. The analysis was carried out according to the procedure previously described(1). Urine was collected for 17 hours, but the results were calculated for 24 hours. If the urine volume during the 17 hour collection period did not exceed 5 cc, the specimen was discarded. Extracts of posterior pituitary gland (P.P.E.) were prepared as previously described by saline extraction of powdered acetone dried tissue. All injections were made in 3 cc of saline intraperitoneally. Parke-Davis pitressin, assaying 20 pressor units per cc, was used. The ACTH was an Armour product (Corticotrophin).

Results. The excretion of NRL by the normal guinea pig was usually between 60 γ-150 γ per 24 hours. However, the excretion for animals tested on the same day was

TABLE I. Excretion of NRL by Guinea Pigs following Administration of ACTH, P.P.E., Pitressin and Pitocin.

Substance	Dose	No. of animals	Mean NRL per 24 hr	Mean excretion ratio
ACTH	8 U	4	.118	1.18 ± .33*
	12	4	.143	1.45 ± .40
	16	4	.224	2.15 ± .97
P.P.E.	.5 mg	4	.097	1.09 ± .33
	1	4	.174	1.71 ± .49
	2	4	.277	3.55 ± .56
Pitressin	.5 U	4	.102	1.24 ± .34
	3	4	.182	1.74 ± .63
	5	4	.192	2.14 ± .25
	5†	6	.182	2.00 ± .48
	4‡	8	.148	1.55 ± .69
	8‡	8	.260	2.00 ± .47
	5◊	8	.181	2.25 ± .65
	5‖	4	.222	2.32 ± .09
Pitocin	5 U	6	.106	.93 ± .25

* Stand. dev.

† Administered in 4% saline.

‡ du Vigneaud preparation assaying 300 units/mg.

◊ Given 8 min. after I.M. inj. of 5 mg of Apresoline.

‖ Given 20 min. after I.M. inj. of approx. 5 mg of Dibenzylene.

* This project was supported by a grant from the Public Health Service.

usually in much closer agreement. The reason for the variation is unknown, and seasonal effects appear to account for only part of this. Consequently, the results of the excretion of animals experimentally treated were expressed as the ratio between the experimental value and the mean excretion of 2 normal animals tested at the same time. For convenience one NRL unit was defined, as the minimum amount of material which when given intraperitoneally into a guinea pig caused a doubling of NRL excretion (Ratio of 2) as compared to the controls.

In Table I are shown the effects of the administration of ACTH, P.P.E. and pitressin. While in many instances numerous experiments were carried out, the results of only several individual experimental groups are shown. One NRL unit was contained in approximately 15-20 units of ACTH, the extract from 1-2 mg of posterior pituitary powder and 2-4 units of pitressin.

The pressor activity of one NRL unit of P.P.E. and pitressin were similar when tested in the rat by the direct intraarterial procedure. Increasing the dosage above the quantities necessary for one NRL unit did not necessarily increase further the NRL activity. For example the NRL response to P.P.E. from 50 mg of powder was not very much greater than an extract from 1-2 mg although individual animals responded with as much as a 4-fold increase in NRL.

Partly purified pitressin (vasopressin) containing 300 pressor units per mg† was tested. One NRL unit corresponded to 8 pressor units. The significance of the discrepancy between pressor dosages required of the commercial and the purified pitressin is not clear. Pitocin did not increase NRL excretion.

To study the mechanism involved in the increased NRL excretion, 5 pressor units of pitressin were used routinely to elicit this response. This dose is well tolerated and consistently gives an NRL assay in excess of one unit. The pressor response following intraperitoneal administration in 3 cc of saline is shown in Fig. 1. The blood pressure rises to

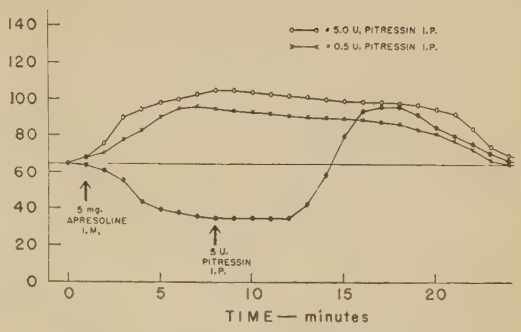

FIG. 1. Pressor response following intraperitoneal administration of 0.5 U pitressin, 5 U pitressin, and 5 U pitressin following 5 mg of Apresoline I.M.

a peak of 115 mm of mercury in 8 minutes and is maintained at an elevated level for an additional 15 minutes. At 8 and 20 minutes after injection, the blood has a pitressin concentration of approximately 50 milliunits per cc. The volume of the overnite collection of urine is usually increased; however an increase in NRL excretion can occur in the absence of an increase in urine volume.

Hyponatremia is observed following the overnite collection of urine in animals receiving pitressin. The serum sodium concentration may fall below 125 m.eq. per l. The fall in the sodium concentration of serum can be prevented if the pitressin is given in a 4% sodium chloride solution instead of physiological saline. Thus 6 animals injected with 5 units of pitressin in 4% saline had a mean serum sodium concentration of 139 m.eq. per l, and the NRL response occurred as usual.

From the data cited above the substance responsible for the NRL increase appears to be pitressin. To eliminate the possibility that this was due to contamination of the pitressin with ACTH, the procedure of Smith and coworkers(7) was employed. These investigators found that when pitressin is heated in a boiling water bath for 30 minutes with 1 N HCl its activity is nearly completely destroyed. When ACTH is heated under these conditions, only a small loss in activity occurs. It was observed that the ability of pitressin to increase NRL excretion was completely destroyed when treated under these conditions.

† Obtained through the generosity of Dr. Vincent du Vigneaud.

Guinea pigs were hypophysectomized by the transpharyngeal approach. All animals were carefully examined for fragments after the completion of the experiment. Hypophysectomy did not decrease the NRL excretion below the normal value. The mean NRL excretion of 7 hypophysectomized animals was 84 $\gamma \pm 42\ \gamma$.‡ After the intraperitoneal administration of 5 U of pitressin to 10 hypophysectomized animals there was no increase in NRL excretion; the mean excretion was 78 $\gamma \pm 39\ \gamma$. Several had a marked decrease in NRL excretion. The mean excretion of 8 animals that still had complete pituitaries or fragments following operation was 88 $\gamma \pm 41\ \gamma$. After 5 U of pitressin they excreted 269 $\gamma \pm 101\ \gamma$. One must conclude from these experiments that pitressin causes an increased excretion of NRL by stimulating the anterior pituitary to release ACTH.

To determine if the increased excretion of NRL depended on the production of systemic hypertension 5 mg of Apresoline were given intramuscularly 8 minutes before the intraperitoneal injection of 5 units of pitressin. The resulting pressure response curve is shown in Fig. 1. The blood pressure fell below normal and rose for a considerably shorter duration and did not rise as high as when 5 units of pitressin alone were administered. The NRL excretion ratio following Apresoline and pitressin administration was not significantly different from that following the administration of pitressin alone. The residual rise in blood pressure is exceeded in duration by 0.5 U of pitressin, a dose insufficient to cause an increase in NRL excretion. This evidence indicates that the establishment of systemic hypertension *per se* is not necessary for the increased NRL excretion. Approximately 5 mg of Dibenzylene, a powerful adrenolytic substance, inhibited completely the pressor activity of 1.5 mg of adrenaline (a near fatal dose for the guinea pig). This dose of Dibenzylene did not inhibit the increased NRL excretion following the administration of 5 units of pitressin.

Arterial and venous blood oxygen contents were unchanged following the administration of pitressin.

Discussion. The evidence herein presented supports previous observations that pitressin can activate the adrenal cortex by causing the release of ACTH. While the possibility of a "stress" reaction can not be ruled out completely the activation of ACTH release is not dependent on systemic hypertension, systemic anoxia, hyponatremia or the release of adrenaline. The evidence is in conformity with the hypothesis that pitressin released by the hypothalamus into the hypophyseal-portal system can activate the anterior pituitary to release ACTH. How this activation takes place is a matter for speculation. It might cause a constriction of the vessels of the hypophyseal-portal system (which are arteriolar§ and could therefore respond to pitressin) and thereby cause a partial anoxia to the anterior pituitary. This could serve as a stimulus for ACTH synthesis or release while it would inhibit the synthesis of other pituitary hormones. Selye(8) has frequently postulated that stress is associated with an increased secretion of ACTH but a decreased secretion of other anterior pituitary hormones.

Summary. Pitressin causes an increased excretion of urinary corticoids by the guinea pig. The presence of the anterior pituitary is necessary for this response. The increased corticoid excretion is not dependent upon the production of systemic hypertension by pitressin, nor is it due to adrenaline release, or the production of hyponatremia, or systemic anoxia. The significance of these findings is discussed in reference to the role of pitressin as a humoral mediator in the hypothalamic activation of the release of ACTH by the anterior pituitary.

The authors are grateful to Neilyn Griggs and Barbara Matecka for their technical assistance, to Dr. P. F. Salisbury for the use of his blood pressure recording apparatus, and to Bernard Schatz for his assistance in operating this apparatus.

‡ Standard deviation.

§ Personal communication from Dr. J. D. Green.

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Action of Thromboplastinase on Human Brain Lipid Fractions.* (21699)

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The mechanism by which tissue thromboplastic suspensions, lipoproteins, and lipids exert their effect in activation of prothrombin is still unknown. The nature of the thromboplastic complex responsible for activity is still controversial. Cohen and Chargaff(1) reported that "lecithin" and "cephalin" showed equal clotting activity. Chargaff(2) later stated that it was impossible to identify the thromboplastic lipid with any of the known phosphatides. Ferguson(3) has shown that clotting activity occurred in several of the Folch sub-fractions of crude brain cephalin. Overman(4) has shown evidence of an activator-inhibitor relationship for the Folch inositol phosphatide fraction. A new approach to these problems employing the action of the bacterial enzyme thromboplastinase has been reported(5,6). The results indicated that TPase was specific for thromboplastically active material of crude tissue extracts and that these extracts might contain a common structural moiety necessary for thromboplastic activity.

This investigation deals with the attempt at further chemical characterization of the thromboplastically active component, employing thromboplastinase action to identify the thromboplastic "lipid" during fractionation.

Materials and methods. Thromboplastinase (TPase) was prepared as previously described(5). 1 mg crude TPase powder/ml water was employed as the standard enzyme concentration. *Thromboplastin*: Unless otherwise noted, human brain and placental thromboplastic suspensions were prepared according to the differential centrifugation method of Chargaff(7). *Crude Thromboplastic Phospholipid*: 200 g of an acetone dehydrated human brain were extracted at 0-5°C for 6 hours with two 500 ml portions of a 1:1 alcohol-ether mixture. Addition of 5 l of cold acetone to the alcohol-ether extract precipitated a crude phospholipid which was removed by centrifugation, dried under nitrogen and stored at –20°C until used. Aqueous suspensions (3% w/v) of this material were prepared by homogenizing in a Teflon homogenizer for 15 minutes at 0-5°C. Prior to use, this suspension was dialyzed against water at 0-5°C for 24 hours to remove all traces of inorganic phosphorus. *Folch lipid fractions*: The sub-fractions of cephalin were prepared according to the method of Folch (8). In all cases, 3% suspensions (w/v) in

TABLE I. Action of "TPase" on Heat Inactivated Placental Thromboplastin (100°).

Time heated, sec	Clotting time, sec	Clotting time after "TPase" action T_{60}	μg P split per mg dry wt
0	11.8	30.0	16.2
15	13.3	31.3	16.5
30	17.1	35.8	16.0
60	20.0	36.0	16.7
120	21.7	38.5	16.3
180	24.4	35.0	17.0
240	26.4	37.0	16.2
300	30.0	37.3	16.3

* This study initiated at the Albert Einstein Medical Center, Philadelphia, Pa., under a grant from the National Institutes of Health.

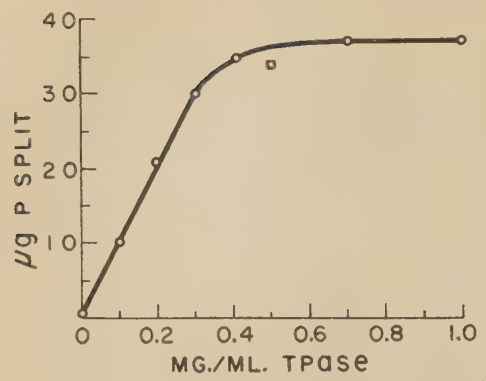

FIG. 1. Phosphorus liberated from crude brain phospholipid as a function of TPase concentration.

water were employed for enzyme studies. *Clotting time determinations*: A modification (5) of the Quick one-stage test was employed to assay clotting potency of all thromboplastic substances used. Unless otherwise noted, normal human oxalated plasma was employed. (9 vol of whole blood to 1 vol of 0.1 M sodium oxalate). *Phosphorus assays*: Phosphorus was determined by the method of King (9) on the supernatant of the incubation mixture obtained after precipitation with cold trichloroacetic acid (10% final concentration). The TCA precipitates were removed by centrifugation at 13,000 r.p.m. for ½ hour.

Results. The action of thromboplastinase on tissue thromboplastic extracts resulted in a loss of clotting potency and a simultaneous liberation of acid soluble organic phosphorus (5). Employing a placental thromboplastic suspension which had been pre-heated at 100°C for varying time intervals, the following became evident: As seen in Table I, heating was effective in decreasing the clotting potency of the substrate but not the thromboplastinase labile phosphorus. The substrate for TPase was apparently heat stable in this system. Loss in activity due to heating and loss due to TPase action were apparently separate phenomena. Similar results were also found with human brain thromboplastin.

Studies employing thromboplastically active crude phospholipid as substrate showed a loss in clotting potency of this substrate as a function of incubation time similar to that reported for tissue thromboplastic substrate(5). The amount of phosphorus liberated in a given time (1 hr) from the crude phospholipid was a function of TPase concentration (Fig. 1). As in the case of intact tissue thromboplastic suspensions(5,6), cupric ion was effective in both inhibiting loss in clotting potency and liberation of organic phosphorus from a lipid substrate (Fig. 2). Per cent thromboplastin destroyed was calculated as described previously(1). Per cent phosphorus liberated was calculated as percentage of amount liberated after a 6 hr incubation period. Complete inhibition of phosphorus liberation could be achieved with higher concentrations of copper but no correlation with clotting activity could be made since higher concentrations of copper inhibited the clotting reaction.

The amount of phosphorus liberated after TPase action on crude lipid substrate was compared with that liberated from the parent acetone dehydrated human brain. To this end, acetone dehydrated human brain was exhaustively extracted with hot 1:1 alcohol-ether. Two fractions were obtained: a crude lipid of low order thromboplastic activity and a thromboplastically inactive residue. The amount of organic phosphorus liberated by

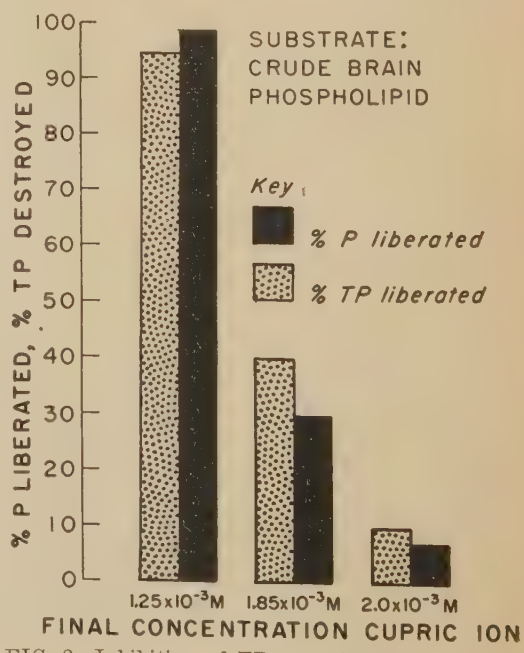

FIG. 2. Inhibition of TPase action by cupric ion.

the enzyme from both fractions was compared with that liberated from the intact acetone dehydrated brain. Although thromboplastinase liberated no phosphorus from the thromboplastically inactive residue, in some cases as much as 50% of total phosphorus of the crude lipid was thromboplastinase labile. However, in no case was the percent of phosphorus liberated from the crude lipid equal to that liberated from intact acetone dehydrated human brain. In a typical case, the liberation of phosphorus from the various fractions calculated as percent of total phosphorus present was as follows: intact acetone dehydrated brain, 66%; active crude lipid fraction, 35%; inactive residue, 0%.

Folch cephalin sub-fractions as well as an alcohol soluble "lecithin" fraction prepared from human brain thromboplastin were tested as substrates for thromboplastinase, using phosphorus liberation as a sensitive quantitative measure of enzyme action, with clotting potency loss serving as a qualitative check. Since the clot accelerator activity of the lipid extracts was found to be low employing the one-stage test, a more sensitive test plasma system was employed as described by Gollub (10). Here, platelet-poor plasma was obtained by high speed centrifugation of blood collected in 1% isotonic Sequestrene® (ethylenediaminetetraacetic acid) in siliconized tubes. The plasma gave a recalcification time of 20-30 minutes.

Activity of each of the lipid fractions was measured at various concentrations in saline. Concentrations showing maximum activity

TABLE II. Activity of Human Brain Lipid Sub-Fractions.

Fraction	Min clotting time, sec	Conc.† (% w/v)
HBTP*	17.7	.75
"Lecithin"	185	.75
Cephalin	106	.30
Inositol phosphatide	95	.50
Fraction II	185	.30
Phosphatidyl serine	160	.75
Fraction IV	286	.03
Phosphatidyl ethanolamine	190	.30

Recalcified clotting time = 1200-1275 sec.

* Human brain thromboplastic powder (acetone dehydrated).

† Initial conc. = 3% (w/v).

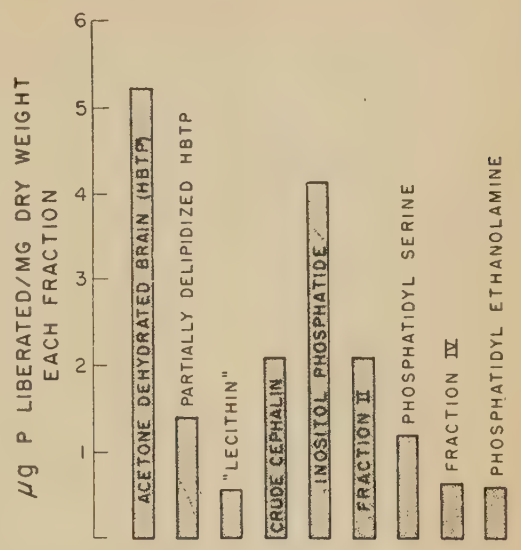

FIG. 3. Action of TPase on human brain fractions.

(minimum clotting time) are indicated in last column in Table II. All lipid fractions showed inhibition of plasma recalcified clotting time at concentrations of 3% (w/v) or higher. However, this level was employed in enzyme studies to standardize substrate concentration.

It may be seen in Fig. 3 that all the Folch lipid fractions had a TPase labile phosphorus fraction, the largest being associated with the inositol phosphatide fraction.

Attempts to identify the liberated phosphorus-containing compound by analysis of the trichloroacetic acid supernatant of both lipid and crude thromboplastic suspensions, have thus far been unsuccessful. No U.V. absorption peak was found. Analysis by the methods of Umbreit(11) for phosphorylated compounds have shown no positive results. Microbiological assays for inositol by the method of Atkin(12), have likewise been negative. A commercially prepared inositol-containing substrate (Lipositol*) has been found inactive with respect to clot accelerator activity and TPase labile phosphorus.

The supernatant did show, however, a 1:1 Kjeldahl nitrogen to phosphorus ratio. The finding of nitrogen in the supernatant after enzyme action on the lipid components is con-

* Bios Laboratory, N. Y. City.

trary to previous reports(5,6) that no nitrogen compound was liberated. Apparently the high content of non TCA precipitable nitrogenous compounds in crude thromboplastic suspensions previously employed masked the liberation of nitrogen due to TPase action on these substrates. A nitrogen-phosphorus-containing material has been recovered from the supernatant after TPase action on both lipid components and the crude thromboplastic extracts as follows: After TPase action, the trichloroacetic acid supernatant was shell frozen and lyophilized. The addition of cold acetone precipitated a fine, white, water soluble powder which showed a 1:1 Kjeldahl nitrogen to phosphorus ratio. Studies are at present underway to identify the components of this material.

Discussion. It appears evident that thromboplastically active lipids, as well as crude tissue thromboplastins, are labile to TPase action showing a loss in clotting potency correlated with phosphorus liberation. Cupric ion inhibits both these effects. The consistently concomitant phosphorus liberation and loss in clot accelerator activity suggests very strongly that these two phenomena are functionally related, and that they are due to the presence of a common structural moiety in all active tissue thromboplastic substances which is labile to TPase action. This moiety seems to contain nitrogen and phosphorus and is associated with the lipid component of crude tissue thromboplastins, although it is not necessarily a lipid. In addition, the results obtained with preheated thromboplastins indicate a heat labile accessory factor possibly protein in nature. It has been shown previously(13), that heat treated thromboplastins did not lose toxicity for rabbits while TPase treated thromboplastins did lose toxicity. Heat treatment also does not affect enzyme labile phosphorus. It would appear then, that toxicity and attachment of the phosphorus moiety are related in some fashion.

The fact that TPase exerted an effect only on thromboplastically active lipids and not on commercially prepared thromboplastically inactive lipids ("cephalin" and "lecithin"(5,6) and lipositol) shows that the enzyme is not capable of attacking phospholipids *per se*. Some specific site of action or compound must be present. These results obtained with cephalin sub-fractions indicate that the Folch fractionation procedure gave an indiscriminate partition of the phosphorus-containing substrate.

It must be noted that, although the action of TPase has been shown to be the same on many crude tissue suspensions(5,6), the results presented above are for human brain components. Lipids prepared from bovine brain have shown similar results. Isolation and identification of the phosphorus-nitrogen material liberated as a result of TPase action may prove of use in elucidating the functional structure of tissue thromboplastins.

The data presented suggest that phosphorus liberation by TPase might prove an accurate method for assay of thromboplastic activity independently of the clotting mechanism. Grossly, the higher the clotting potency, the higher the phosphorus liberation when two thromboplastins are compared. This would be a novel approach in so far as present methods of assay rely entirely on nonspecific nitrogen assay and fibrinogen conversion. This suggestion must be considered with caution, especially in the case of lipid material where higher concentrations of lipid have shown inhibition of the clotting system while incubation with TPase resulted in high phosphorus liberation.

Summary. The action of the bacterial enzyme thromboplastinase on thromboplastically active lipid material has been presented. In all cases, loss in clotting potency was accompanied by a liberation of acid soluble organic phosphorus. A still unidentified phosphorus containing compound has been isolated from the reaction mixture after TPase action. The results are discussed and several possible implications presented.

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Toxin Produced by *Malleomyces pseudomallei.** (21700)

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(Introduced by R. S. Muckenfuss.)

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In studying the pathogenicity of *Malleomyces pseudomallei,* it was frequently noted that mice and hamsters which died within one or 2 days after inoculation of viable organisms showed no gross lesions. This observation suggested the possibility that this organism produced a lethal toxic factor. Legroux, Kemal-Djemil and Jeramec(1) reported that 2 guinea pigs injected intraperitoneally with "lytic filtrate" of broth cultures of *M. pseudomallei* lost weight rapidly. Six days after the first injection, they were again injected with 2 ml of the "lysate." Both animals were dead 48 hours later.

The observations in our laboratory, along with the single experiment reported by the French workers, suggested the search for toxic factors in cultures of *M. pseudomallei* which might account for acute death with only minimal pathological changes in animals experimentally infected with various strains of this organism. This report deals with preliminary observations on the toxicity of bacteria-free filtrates of broth cultures.

Materials and methods. Cultures in beef extract broth containing glycerin in a final concentration of 4% and adjusted to pH 6.8 were incubated statically at 37°C for varying periods of time from one to 12 days. Sterile filtrates were obtained by using Seitz EK, fritted glass UF or Selas 02 and 03 filters. Filtrates which were not sterile were refiltered either through the same or another type of filter. Sterility was determined by inoculating 1 ml of filtrate into 10 ml of glycerin beef extract broth and incubating at 37°C for at least one week. The lethal effect was determined by inoculating Namru mice, 5-10 weeks old, intravenously with 0.4 ml of undiluted filtrate and hamsters, 5-8 weeks old, intraperitoneally with 1.0 ml. Cutaneous reactions were studied in mature albino guinea pigs, approximately 6 months old, by intradermal inoculation of 0.1 ml of various filtrates at widely separated sites.

Results. The accompanying table shows the toxic effect in both mice and hamsters of sterile culture filtrates of *M. pseudomallei,* recently isolated from fatal human cases. The filtrates with the highest toxicity have been obtained with strain 111-4, a rough white isolate from the parent culture which showed both rough white and rough yellow colonies, and strain 118 which also showed both rough white and rough yellow colonies.

The filtrates of strain 111-4, Exp. No. 1 and 4, killed mice in less than 24 hours after inoculation while that in Exp. No. 2 killed only after an interval of 24 hours or more.

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The opinions contained in this report are not to be construed as reflecting the views of the Navy Department or the Naval Service at large (Article 1252, U. S. Navy Regulations, 1948).

TABLE I. Toxicity of Sterile Culture Filtrates of *M. pseudomallei*.

Strain No. and colonial morphology	Organisms/LD_{50}*		Age of culture at time of filtration, days		Filters	Toxicity of filtrates in:‡		
	Mice, ip	Hamsters, ip	Exp. No.			Mice, .4 ml, iv	Hamsters 1 ml, ip	Signs of toxicity in survivors
111–4 Rough white	ca 10^7	< 6	1	10	Seitz EK + UF†	8 D <16 hr 0 S	2 D_1 1 D_3 0 S	
			2	12	Selas 02 + 03 + 03	3 D_1 1 D_2 1 S	2 S	Day 1—Hind leg paralysis, 1 with wet eyes 6—Hind leg paralysis 13—None
			4	11	Selas 03	3 D <20 hr 2 D 22 hr 1 D_3 0 S	1 D_1 1 S	1—Wet eyes 6—Large skin lesion at site of inoculation 13—None
112 Smooth with few mucoid colonies	ca 10^4	<47	1	8	Seitz EK + UF	1 D 20 hr 1 M_1 (killed) 2 S	2 S	1—Hind leg paralysis 6—None
			4	11	Selas 02 + 03	1 D_2 3 S	2 S	1—One none One with hind leg paralysis & ocular discharge 6—None
118 Rough white and rough yellow	$<10^3$		9	12	Selas 03	8 D <18 hr 0 S		

* Avg of previous titrations on the stock cultures. † Fritted glass filter of UF porosity. ‡ 3 D_1 = 3 dead within 1 day, etc. S = survived; M = moribund.

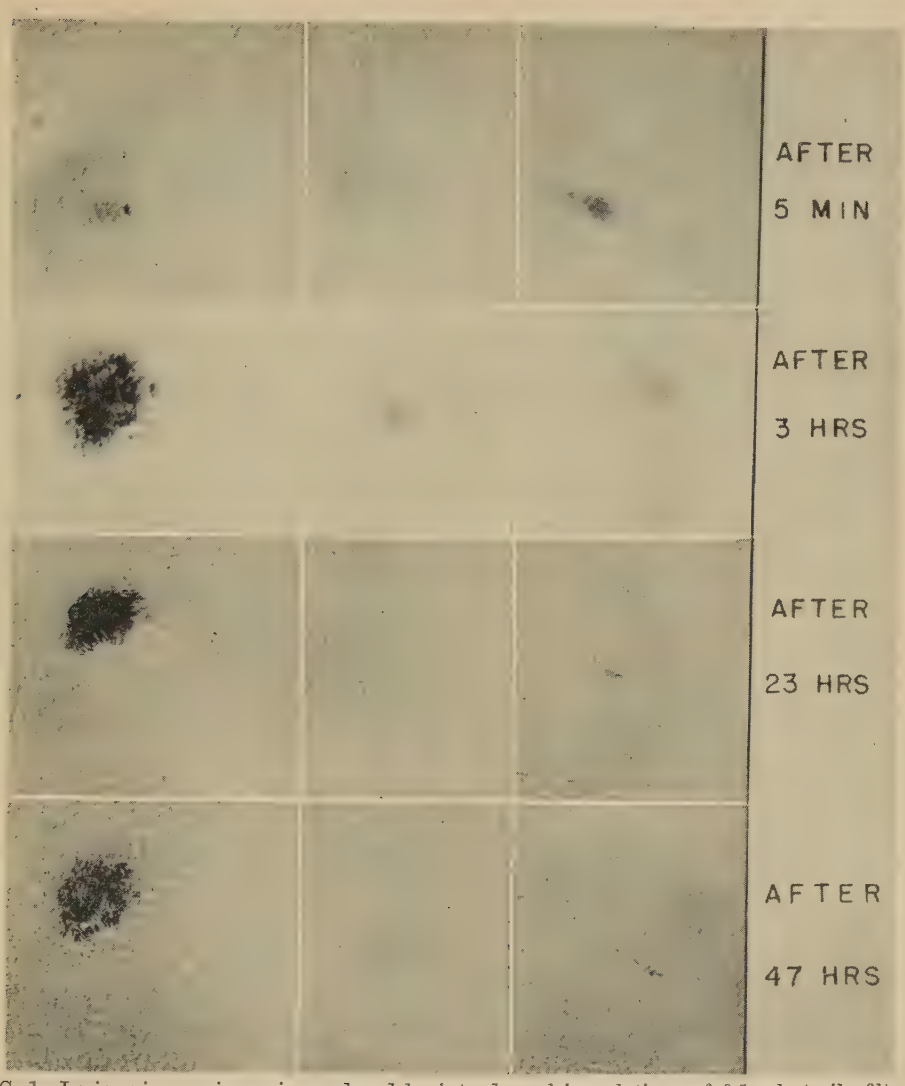

FIG. 1. Lesions in a guinea pig produced by intradermal inoculations of 0.1 ml sterile filtrate of an 11-day-old broth culture of *Malleomyces pseudomallei*, strain 111-4. Unheated filtrate was inoculated at the left and boiled filtrate at the right. Uninoculated culture medium was inoculated in the center.

The 3 successive filtrations through Selas candles, necessary to obtain a sterile filtrate in this instance, may account for its lower activity. The filtrate of strain 118 killed all of 8 mice in less than 18 hours.

While the data shown in the table indicate that strain 112 was less toxigenic than strain 111-4, the 112 filtrate of Exp. No. 4 after the first filtration through the Selas 02 filter killed 3 out of 4 mice in less than 24 hours. Although the sterility tests after this first filtration showed a few viable organisms, the latter could hardly account for the early deaths observed since mice inoculated with approximately 100,000 organisms of this strain did not die of infection until 4 days or more after inoculation. The subsequent filtration through a Selas 03 filter apparently resulted in the loss of considerable activity.

The toxicity in hamsters inoculated intraperitoneally with one ml of the various filtrates was parallel to that in mice. While hamsters were not as readily killed as mice under the test conditions, those which were

not killed nevertheless developed profound signs of toxicity from which they gradually recovered quite completely. The most striking sign was paralysis of the hind legs and excessive lachrymation, with eyes closed. The mice which survived showed similar signs of toxicity.

The effect of intradermal inoculation of heated and unheated filtrates in guinea pigs is shown in Fig. 1. Within 5 minutes after inoculation, the unheated preparation had already produced deep reddening of the skin and within 15 minutes a hemorrhagic lesion became evident. The hemorrhagic lesion was fully developed within 3 hours. Within 6 hours there was definite erythema and edema which became marked within 24 hours. The reactions were maximal within 48 hours. By the 6th or 7th day, the edema and erythema had nearly subsided, leaving only a healing necrotic center.

In contrast to the unheated filtrate, the boiled preparation did not produce the immediate hemorrhagic lesion but after 6 hours, there was some erythema and edema which increased during 48 hours and was comparable to that produced by the unheated preparation. By the 7th day the lesion had almost healed.

The results of the intradermal tests indicate that there were at least two toxic components in the filtrates. Heating at 100°C inactivated the component responsible for the immediate hemorrhagic lesion whereas the component responsible for the delayed non-hemorrhagic lesion was not significantly affected by boiling for 10 minutes.

Preliminary observations indicate that the toxic factor accumulates slowly in the growing culture, since filtrates from 24 hour and even 4 day cultures, with viable counts of 10^9 and 10^{10} organisms per ml respectively, showed little or no toxicity. The toxic factor may be an endotoxin which increases quantitatively as autolysis progresses. Cultures incubated statically appeared to be more active than parallel cultures incubated for the same length of time on a reciprocating shaker.

From all the work to date there appears to be no correlation between colonial morphology and toxicity. The colonial morphology of strain 111-4 was rough white; that of strain 112 was largely smooth, with a small percentage of mucoid colonies, while strain 118 showed both rough white and rough yellow colonies.

Some of the mice which survived the inoculation of filtrates of varying potency were held for 2 to 4 weeks at which time they were challenged with a bacterial suspension of a mouse adapted smooth strain of *M. pseudomallei.* Twelve of the 18 survivors from these various experiments which were thus challenged survived an infecting dose (approximately 5 LD_{50}) which killed all of the control mice within the 14 day observation period. These results suggest that detoxified toxic filtrates might produce substantial immunity against infection.

Summary 1. *Malleomyces pseudomallei* produced in broth cultures a toxin which was lethal for both mice and hamsters. 2. Intradermal inoculation of bacteria-free filtrates of broth cultures of *Malleomyces pseudomallei* produced an edematous, erythematous and hemorrhagic lesion in normal guinea pigs. Boiled filtrates produced only delayed erythema and edema, suggesting the presence of a thermolabile component which was responsible for the hemorrhagic lesion. 3. The potency of the toxin did not appear to be related to the colonial morphology.

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Pharmacological Properties of Canescine ('Recanescine'*), a New Alkaloid Isolated from *Rauwolfia canescens* Linn. (21701)

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The alkaloids which have been isolated from *Rauwolfia serpentina* and other species of Rauwolfia can be classified into 3 groups according to the character of hypotensive and other pharmacological responses induced in experimental animals. The first, heretofore represented by reserpine and rescinnamine, produces a characteristically delayed, but long lasting hypotension, accompanied by bradycardia and sedation. There is no inhibition of the pressor response to epinephrine. The second group consists of alkaloids which produce yohimbine-like effects(1), such as a precipitous, but short-lasting hypotension and an epinephrine reversal. The third comprises a miscellaneous group of alkaloids that are essentially devoid of cardiovascular or sedative activity.

Recently, Klohs and co-workers isolated a new, highly potent alkaloid, 'Recanescine,' from *Rauwolfia canescens* Linn.(2). It is the trimethoxybenzoic acid ester of an alkamine, which is probably 11-desmethoxymethyl-reserpate. As will be shown in this communication, Recanescine is a member of the same pharmacological group as reserpine and rescinnamine.

Methods. Recanescine was tested in dogs at the end of 5 days of oral medication or acutely following intravenous injection in urethanized animals by procedures described previously(3). Sedative effects were determined quantitatively in mice by measuring prolongation of pentobarbital-induced sleeping time(3) and in roller cages described by Young and Lewis(4). For the latter test, the alkaloid, dissolved in dilute acetic acid, was injected intraperitoneally and 2 hours later the animals were placed in the roller cages for one hour. Results were expressed as percent "fall-outs." The protective effect of Recanescine against amphetamine was investigated by determining the intraperitoneal one hour LD_{50} of amphetamine sulfate in mice five hours following intraperitoneal injection of the alkaloid. In another experiment, amphetamine sulfate was given intraperitoneally at the constant dose of 100 mg/kg which had been found in 170 mice to cause a mortality of 80% within one hour. Recanescine was injected intraperitoneally in varying doses 5 hours before the amphetamine and the dose was calculated which reduced mortality from 80% to 40% (PD_{50}).

Results. The effects in dogs of repeated oral administration of Recanescine ranging from 1 to 320 γ/kg were bradycardia, hypotension and sedation. (Table I). All these effects were dose dependent over the tolerated dose range. The highest amount used (320 γ/kg/day) killed 2/5 dogs, one each on the 3rd and 4th day respectively. Nictitating membrane prominence, myosis and diarrhea were frequently observed. Sedation was observed grossly and was also evidenced by a reduction of the amount of pentobarbital sodium required to induce anesthesia, as has been reported for rescinnamine(5). The results obtained with Recanescine are very similar to those observed after the same doses of reserpine(6).

The characteristic latency in onset of action of reserpine and rescinnamine was also shown by Recanescine. When given intravenously to four urethanized dogs in doses of 100-250 γ/kg, 3 to 6 hours elapsed before MAP had fallen significantly, reaching values in the range of 62-110 mm Hg as compared to pre-drug MAP of 116-150 mm Hg. (For control values see ref. 3). The primary pressor response to central vagal stimulation in bilaterally vagotomized animals had completely disappeared in all four cases 150-210 minutes after the drug. The response to bilateral carotid occlusion after 6 hours was

* Trade-mark name of Riker Laboratories, for its canescine alkaloid.

TABLE I. Observations in Dogs after 5 Daily Oral Doses of Recanescine.

Dose, γ/kg	No. of dogs	HR*	MAP*	Sed.†	NM†	Dia.†
Controls	54	135 ± 24	113 ± 20	0/54	0/54	0/54
1	10	110 ± 28	111 ± 22	0/10	1/10	1/10
3.2	9	108 ± 34	91 ± 18	2/ 9	5/ 9	2/ 9
10	10	116 ± 18	92 ± 26	1/10	4/10	1/10
32	10	103 ± 27	83 ± 17	2/10	5/10	3/10
100	10	80 ± 17	75 ± 19	5/10	9/10	7/10
320	3	88 ± 15	81 ± 10	3/ 3	3/ 3	3/ 3

* HR (heart rate in beats/min.) and MAP (mean arterial pressure in mm Hg) measured under pentobarbital and expressed as mean ± stand. dev.

† Animals showing sedation (Sed.), nictitating membrane prominence (NM), or diarrhea (Dia.)/animals tested. These data were recorded before anesthesia was induced.

decreased, both absolutely and in relation to existing MAP. The pressor response to epinephrine was not blocked either 15 minutes or 5 hours after the drug.

Pentobarbital-induced sleeping time was prolonged significantly in mice (Table II) after 0.5 mg/kg of Recanescine, and it was increased with increase in dosage. In this respect, Recanescine was found to be considerably more potent than rescinnamine(6) and of the same order of activity as reserpine.

The "fall-outs" of mice in the roller cage experiments indicated a very significant lowering of threshold after 1 mg/kg of Recanescine. Of 234 control mice 14, or 6%, fell out within 1 hour. Thirty to 45 mice each were injected with the following dosages of Recanescine with the corresponding percent "fall-outs"; 0.5 mg/kg, 11%, 1 mg/kg, 48%, 2 mg/kg, 77%, 4 mg/kg, 90%. The ED_{50} (with 95% C.L.) calculated according to Litchfield and Wilcoxon was 1.2 (0.8-1.8) mg/kg. Using 10 animals at each of 5 dosages the protective effect of Recanescine against toxic doses of amphetamine sulfate was indicated by an LD_{50} (with 95% C.L.) of 152 (143-161) mg/kg when determined 5 hours after intraperitoneal injection of 2 mg/kg of the alkaloid as compared to LD_{50} of 90 (83-97) mg/kg for amphetamine sulfate alone (10-20 animals at each of 8 dosages). The PD_{50} following a constant dose of 100 mg/kg of amphetamine sulfate was 0.045 (0.034-0.060) mg/kg of Recanescine, given intraperitoneally 5 hours prior to the test. (10 animals at each of 10 dosages of Recanescine).

TABLE II. Effect of Recanescine on Pentobarbital Induced Sleeping Time in Mice.*

Dose, mg/kg	No. of groups, 10 mice each	Sleeping time, % of controls
0.25	3	115 ± 38
0.5	3	158 ± 15†
1.	4	161 ± 25†
2.	4	217 ± 13†
4.	4	255 ± 37†
8.	3	268 ± 90†
16.	3	289 ±110†

* Recanescine given intraperitoneally 2 hr before standard dose of pentobarbital sodium (30 mg/kg intraperitoneally).

† Significant increase ($p = 0.05$ or less).

Discussion. The new alkaloid Recanescine has the typical pharmacological properties of reserpine and rescinnamine. It produces bradycardia, hypotension and sedation, and it is free from adrenolytic (yohimbine-like) properties. In potency, it seems similar to reserpine and rescinnamine. It has been isolated from *Rauwolfia canescens*, but its distribution among other Rauwolfia species is still being investigated. It is yet another alkaloid which may contribute directly to the therapeutic usefulness of Rauwolfia preparations. No evidence of differences in the mechanism of action between Recanescine, rescinnamine and reserpine has been observed.

The mechanism which raises the tolerance of mice to lethal doses of amphetamine is not known. However, since this effect is related to the dose of Recanescine and, since this alkaloid as well as reserpine and rescinnamine effectively suppress also the hypermotility caused by amphetamine, it is suggested that the protective effect against amphetamine is related to the tranquilizing ("sedative") properties of these alkaloids.

Summary. The pharmacological properties

of Recanescine, a new alkaloid recently isolated from *Rauwolfia canescens,* have been found to be similar to reserpine and rescinnamine. Recanescine produces hypotension, bradycardia and sedation accompanied by characteristic alterations in cardiovascular responses. These are depression of the pressor response to bilateral carotid occlusion and blockade or reversal of the pressor response to central vagal stimulation. The sedative effects of Recanescine have been observed grossly in dogs and mice, and they have been demonstrated objectively in mice by prolongation of pentobarbital induced sleeping time and by increased "fall-outs" from roller cages. Recanescine significantly raised the tolerance of mice to toxic doses of amphetamine. This has been interpreted as due to the sedative effect of this alkaloid.

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Observations on Bacterial Interaction.* (21702)

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Lactobacillus bifidus is of interest as the characteristic flora of the intestine of the normal breast-fed infant. A variant of *L. bifidus* which requires for its growth a factor present in human milk but only in traces in cow's milk has been repeatedly isolated in this laboratory(1). The "Bifidus Factor" in human milk was found to be an oligosaccharide containing N-acetyl-D-glucosamine(2). It is considered probable that this variant (*L. bifidus* var. *pennsylvanicus*) is the parent organism which may mutate into the non-milk-requiring regular form of *L. bifidus*.

In routine use of *L. bifidus* var. *pennsylvanicus* as test organism for studying the activity of various materials as Bifidus Factor, an occasional assay was invalid because of an inconsistent growth curve characterized by lighter growth in the presence of small amounts of the Bifidus Factor than in its complete absence. It was found that this response could be explained by the presence of a mutant in the culture. Further studies of the mutual effects of the variants of *L. bifidus* were made, as well as of the effect of these organisms on the growth of *Escherichia coli.*

Methods. The medium and methods used in the culture of regular *L. bifidus* and of *L. bifidus* var. *pennsylvanicus* have been described(1). Growth has been estimated by titration of the acid produced and by turbidimetric measurements in the Klett-Summerson photometer. In a number of the experiments 2 organisms were grown together in the same tube. In other cases, to measure growth of the organisms separately while allowing mixture of metabolic products, dialysis using bags of viscose tubing was employed. Inoculum of one strain was put into the viscose tube, and of the other strain into the outer glass tube. Control tests of the strains separately were handled in the same way. In experiments with *Escherichia coli* the amount of sodium acetate in the bifidus medium was reduced from 2.5% to 0.5% since the usual concentration of the salt was found to be toxic for the *E. coli.* The double-strength medium was Seitz-filtered and added aseptically to the tubes since sugar autoclaved in the medium is

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TABLE I. Growth of *L. bifidus* var. *pennsylvanicus* with Varying Amounts of Skimmed Human Milk.

Human milk (ml/10 ml medium)	Acid production (ml 0.1 N/10 ml culture) Normal curve	Abnormal curve
.0	1.3	6.4
.006	2.5	4.0
.02	6.0	4.3
.06	12.1	10.8
.2	18.5	16.1

not satisfactory for growth of *E. coli* while this medium, autoclaved without the sugar, is somewhat toxic to *L. bifidus*. The strain of *E. coli* was 0111 B4. It was carried in nutrient broth. An 18 hour culture was used as inoculum.

Results. The two growth curves shown in Table I were obtained from 2 successive subcultures of *L. bifidus* var. *pennsylvanicus,* 212 a-s-3. The first is of the normal form for the organism. The second illustrates the type of aberration which initiated this investigation. The high acid production in the tube without supplement of human milk suggested that a non-milk-requiring mutant was present. When cells from this tube or from the inoculum used were streaked out on a plate with regular medium supplemented with 2% of skimmed human milk, 2 types of colonies were observed: relatively large white colonies and smaller gray colonies. The former are typical of the regular strains of *L. bifidus,* the latter of the milk-requiring strains.

If growth in the absence of bifidus factor is due to the presence of a mutant strain, the decrease in acid production in the presence of small amounts of human milk may be attributed to repression of the mutant. No indication was found that growth of the regular strains of *L. bifidus* was inhibited by human milk: in consequence the growth of the mutant must have been inhibited by the parent strain itself. To determine which type of *L. bifidus* predominated at each level of bifidus factor used inocula from all of the tubes of a growth curve were plated out on regular medium and on medium supplemented with 2% of skimmed human milk. The results of one test are shown in Table II. On the plates without supplement of human milk (which would not support growth of *L. bifidus* var. *pennsylvanicus*) white colonies were present throughout, the number of the colonies decreasing with the amount of bifidus factor which had been present in the assay tube. With the plates supplemented with human milk, white colonies were found only on that inoculated from the tube containing no bifidus factor. As would be expected, the number of colonies of *L. bifidus* var. *pennsylvanicus* increased with increasing amounts of bifidus factor in the assay tube. Thus, in the tubes, the growth of *L. bifidus* was inversely proportional to that of *L. bifidus* var. *pennsylvanicus*.

The observations made on the mixed culture which was obtained by spontaneous mutation were confirmed by tests with mixtures of a standard strain of *L. bifidus* (Jackson, single cell(3,4)) with a strain of *L. bifidus* var. *pennsylvanicus* which showed no measurable tendency to mutate. Using a mixed inoculum with a ratio of *L. bifidus* var. *pennsylvanicus* to *L. bifidus* of 100,000 there was growth in the tube without supplement of human milk. The regular strain grown alone from an inoculum of the same size produced 3 times as much acid as when it was grown in the presence of *L. bifidus* var. *pennsylvanicus* even though the latter grew only slightly (alone it produced only 0.6 ml of acid and changed the pH of the medium from 6.52 to 6.25).

In another experiment the inoculum of *L. bifidus* was separated from that of *L. bifidus* var. *pennsylvanicus* by a viscose membrane so that the growth of each strain could be esti-

TABLE II. Proportion of *L. bifidus* in Cultures of *L. bifidus* var. *pennsylvanicus* in Medium Containing Varying Amounts of Bifidus Factor.

Human milk (ml/10 ml medium)	Acid production (ml .1 N/10 ml culture)	Colony type on plates* Regular medium	Medium with 2% human milk
.0	13.1	White+++	White+++
.006	11.2	" ++	Gray +
.02	6.0	" +	" +
.06	11.7	" +	" ++
.2	17.9	White+, Gray +	" ++

* Both types of colonies could be observed on all plates, but only the one indicated in appreciable number.

mated turbidimetrically. In the absence of bifidus factor there was no depression of growth of the regular strain. However, in the tube containing 0.06% of skimmed human milk the growth of the regular strain was reduced by about 30% while with 0.6% skimmed human milk, the depression of growth of the regular strain was over 80%.

Later experiments were carried out in medium containing 2% human milk, the most favorable conditions for the growth of *L. bifidus* var. *pennsylvanicus*. Each strain was used at 3 levels of inoculum, covering a 100-fold range of concentration. For *L. bifidus* var. *pennsylvanicus* the highest concentration was that usually used in assay, while the non-milk-requiring *L. bifidus* was given the advantage of a heavier inoculum with 10 times the usual amount as the highest level. Under these conditions also the presence of *L. bifidus* var. *pennsylvanicus* markedly inhibited growth of *L. bifidus*. Even when the inoculum density of *L. bifidus* var. *pennsylvanicus* was one-tenth that of *L. bifidus*, growth of the latter was less than 40% of that attained when the strain was grown alone. *L. bifidus* had a relatively slight effect on *L. bifidus* var. *pennsylvanicus*. Only when the concentration of inoculum of *L. bifidus* was 100 to 1000 times greater than that of *L. bifidus* var. *pennsylvanicus* was there appreciable inhibition of the latter.

A number of experiments were made to determine whether *L. bifidus* and particularly *L. bifidus* var. *pennsylvanicus*, exerted an inhibitory effect on the growth of *E. coli*. The medium designed for the growth of *L. bifidus* was not optimal for the growth of *E. coli*, even after the amount of sodium acetate was reduced. Lactose, which is particularly desirable for *L. bifidus* was not as satisfactory as glucose for *E. coli*. On the basis of turbidimetric measurements *E. coli* attained a concentration of cells less than 20% of that reached by *L. bifidus*. When *E. coli* was grown with regular *L. bifidus* the growth of the former was stimulated at almost all levels of inoculum chosen. Apparently some metabolic products of *L. bifidus* enriched the medium for *E. coli*. One factor may have been glucose which is produced by the lactase of *L. bifidus*. In contrast, there was no stimulatory effect of *L. bifidus* var. *pennsylvanicus* on *E. coli*. Using a light inoculum of *E. coli*, *L. bifidus* var. *pennsylvanicus* exerted definite inhibition in comparison with the growth of *E. coli* alone. In all instances the growth of *E. coli* was remarkably lower than when it was grown with the regular *L. bifidus*.

Both types of *L. bifidus* and *E. coli* were grown in culture filtrates from the regular and milk-requiring strains of *L. bifidus* to see if an inhibitory agent was present in these culture filtrates. The cultures were incubated for various periods of time; the pH of the medium was adjusted to that of the original medium and the filtrates were Seitz-filtered. No evidence could be obtained by this method for the accumulation of a specific inhibitor in the filtrates from *L. bifidus* var. *pennsylvanicus*.

Discussion. *L. bifidus* var. *pennsylvanicus* was isolated in the course of studies on the effect of human milk on the growth of *L. bifidus*. There had been previous evidence that mutation to a non-milk-requiring form occurred. If the organism began to show "high blank," that is an acid production of 2-3 ml of 0.1 N acid per 10 ml rather than the usual 0.5-1 ml in tubes without supplement of Bifidus Factor, the stock culture was plated out and subcultures were made. The present study suggests how an organism which is so commonly present in infant feces could so long escape detection. If the stool was inoculated on to a plate or tube furnishing all of the essential nutrients except the specific "bifidus factor," the mutant cells, though only a minute proportion of the total population could multiply much more rapidly than the normal cells and become dominant. On the other hand, if the culture is supplied with an excess of bifidus factor the parent strain has the advantage and the mutant strain is inhibited. No specific inhibitory action of the parent strain would be required to bring about this result, although the present study suggests that *L. bifidus* var. *pennsylvanicus* does have such an action on the usual type of *L. bifidus* and can prevent its multiplication even when, itself, growing only slightly.

The relationship between *L. bifidus* var.

pennsylvanicus and regular *L. bifidus* is similar to that observed by Ryan and Schneider (5-8), with a histidine-requiring strain of *E. coli* and a histidine-independent mutant. The preponderance of one or the other strain was found to be dependent not only on the histidine content of the medium at any time but on other factors such as the hydrogen ion and glucose concentrations. The histidine-requiring strain, even after multiplication had stopped because of depletion of histidine, continued to metabolize glucose and so modified the rate of growth of the histidine-independent strain. These authors discuss the probability that many mutations which have been classified as mutation by adaptation may be mutation by suppression of the parent strain by environmental conditions. This type of interaction must be operative in a mixed flora such as that of the intestinal tract. No conclusions can be drawn from the *in vitro* tests reported here as to the relationship of *L. bifidus* var. *pennsylvanicus* to *E. coli* in the intestine of infants. They do suggest how a well-established *L. bifidus* var. *pennsylvanicus* flora might suppress the growth of less desirable organisms.

Summary. An inhibitory effect of *Lactobacillus bifidus* var. *pennsylvanicus* on *Lactobacillus bifidus* has been demonstrated. A similar effect of *L. bifidus* var. *pennsylvanicus* on *Escherichia coli* was indicated.

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Hemodynamic and Metabolic Response of Human Hypertensive Kidney to Sodium p-Sulfamyl Benzoate.*† (21703)

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Studies of renal oxygen consumption have shown that this factor is a relatively fixed quantity despite the introduction of various stresses(1-4). Only the presence of certain renal diseases(5,6), abdominal compression (7), and the utilization of salt poor human albumin(8), have been capable of altering this function.

Previous *in vitro* and *in vivo* studies(9-11), have demonstrated that p-sulfamyl benzoic acid (Dirnate ®) or its sodium salt is capable of inhibiting carbonic-anhydrase activity of the distal tubule. Because of the specificity of this action it appeared to provide an additional agent for the furtherance of studies designed to test the effects of tubular enzymatic blocking agents on the renal oxygen consumption. The following data are the results of the action of sodium p-sulfamyl benzoate on the hemodynamics and metabolism of the human hypertensive kidney.

Materials and methods. Six hypertensive patients of Smithwick grade II-IV were studied in a fasting state. The selection of patients with hypertension was prompted by the suggestion that such an agent might be of value in the maintenance of a low sodium re-

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† Sodium p-Sulfamyl Benzoate (Dirnate®) supplied by Sharp and Dohme, West Point, Pa.

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gime as part of their therapy. Measurements of *glomerular filtration* rate, PAH extraction, hematocrit, total renal blood flow, arterial-renal venous oxygen difference, and the calculation of renal oxygen consumption were made according to previously described technics(2). Control observations represented the mean of 2 fifteen minute periods before the intravenous administration of 2 g of sodium p-sulfamyl benzoate. Experimental studies were the mean of 2 fifteen minute periods taken 30-45 and 45-60 minutes after the injection of the drug. The selection of these times was based on the observation that a relatively "steady state" with regard to the effect of this agent on electrolyte excretion existed at these times(12). Urinary pH was measured in 5 of the 6 subjects by means of pHydrion Test Paper.§ In all patients mean arterial blood pressure was determined(2) and in 5 patients cardiac output (Fick) was measured immediately before and after the renal study. The tubular maximal secretory capacity for PAH (Tm_{PAH}) was determined simultaneously in one patient.

Results. The intravenous administration of 2 g of sodium p-sulfamyl benzoate resulted in a variable response in urine flow and no change in hematocrit. A significant rise in urinary pH (Mean = 2.3 units, $p = <0.01$) was noted in the 5 patients in whom this was measured and was due to an increased excretion of sodium and potassium(12).

In contrast to these marked changes in excretory function, glomerular filtration rate ($\Delta\%$‖ −11, $p = >0.2$), total renal blood flow ($\Delta\%$ −1, $p = >0.8$), the arterial-renal venous oxygen differences ($\Delta\% = +1$, $p = >0.8$), the renal oxygen consumption ($\Delta\% = 0$) and Tm_{PAH} ($\Delta\% = -6$) showed no significant change. A significant decrease in the extraction of PAH ($\Delta\%$ −6, $p = <0.05$) was probably due to a competition between the agent under study and PAH for the same excretory mechanism since the former is also secreted by the proximal tubule although its action is presumably in the distal tubular segment(10).

Although 5 of the 6 patients showed a decrease in mean arterial blood pressure these changes were not significant ($\Delta\% = -6$, $p = >0.3$). Cardiac output increased in 2 patients and fell in 3 such that the net result for the group was not statistically significant ($\Delta\% = -3$, $p = >0.05$).

Discussion. Enhancement or inhibition of proximal tubular function by PAH(1) and Probenecid(4), respectively, produces no change in renal oxygen consumption. Likewise inhibition of distal tubular function by mercurial diuretics or, as shown above by a carbonic-anhydrase inhibitor, results in no alteration in this function. However all of these agents produce an increase in urinary solutes and therefore an increase in urinary osmolarity(12,13).

Since the employed methods are capable of determining changes in renal oxygen consumption(7,8) these results might be explained on the basis that the decrease in oxygen utilization associated with the inhibition of one system is exactly counter-balanced by an increase in oxygen consumption by means of the enhancement of another system. Although such an explanation might be tenable in the case of sodium p-sulfamyl benzoate where an inhibition of hydrogen ion secretion is associated with an enhanced secretion of potassium it is rendered unlikely in view of studies with Probenecid(4) which is purely inhibitory in action but which produces no change in oxygen utilization.

An alternative, and the most plausible, explanation for the findings with sodium p-sulfamyl benzoate is that the kidney utilizes oxygen for the maintenance of its cellular integrity and so long as the latter is maintained there is no change in oxygen consumption regardless of changes in excretory function. Further support for this concept is found in the papers of Bradley(7), Conn *et al.*(15) and Crosley *et al.*(6), which demonstrate a direct correlation between renal oxygen consumption and functional tubular mass.

Summary and conclusions. Intravenous administration of 2 g of a carbonic anhydrase inhibitor, sodium p-sulfamyl benzoate, pro-

§ pHydrion Test Paper—Micro Essential Laboratory, Brooklyn.

‖ $\Delta\%$ = Mean change of group from individual mean control values.

duced no significant changes in renal hemodynamics or metabolism, mean arterial blood pressure or cardiac output. The negativity of these results despite large changes in urinary pH and electrolyte excretion(12) provide additional support to the concept that the kidney utilizes oxygen for the maintenance of cellular integrity rather than for the performance of external work.

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Effect of Whole-Body X-Irradiation on Uptake of Iron by Duck Erythrocytes.* (21704)

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The blood cells of the duck *in vitro* take up iron added to blood(3,4,5). After phlebotomy the iron uptake and the reticulocyte level increase and return to normal in parallel(4). In contrast to the bird cells, those of normal mammals take up but little iron(3,4,6). When, however, the level of reticulocytes is increased, the uptake becomes marked(4,6). It is fairly certain, therefore, that the additional reticulocytes appearing in blood after certain stimuli remove iron from plasma. However, it is less certain which cells in normal blood take up added iron.

In the present work further test was made to determine which blood cells account for the uptake of iron *in vitro*. For this purpose, the uptake was measured in the duck after whole-body x-irradiation, which was expected to produce change in the numbers of blood cells, at least. A decrease in uptake was found that appears explained by a decrease in numbers of one class of reticulocytes.

Methods and materials. Uptake was measured as follows: 14.6 millimicromoles of radioactive ferric iron (Fe 59) in 0.067 ml of 0.1 *M* sodium citrate were added per ml of blood or cell preparation. One ml of the mixture was incubated 4 hours at 37° under room air and then centrifuged. The plasma was removed. The cells were washed with 2 ml of 0.156 *M* sodium chloride. The radioactivity of the plasma plus the washing and of the cells was measured by means of a scintillation counter. The value of the ratio *activity in the cells/activity in the cells + activity in the plasma plus washing* was calculated and is denoted uptake. *Erythrocytes* were characterized as follows: On each of 2 cover slips

* Preliminary reports(1,2). Supported by the U. S. Atomic Energy Commission.

was placed a small portion of a mixture of blood and 0.156 *M* sodium chloride, 1 and 3 parts respectively. The cover slips were placed on a slide coated with brilliant cresyl blue (several drops of a solution, 0.1 g plus 10 ml absolute ethanol, were spread on the slide and allowed to dry.) After the diluted blood between slide and slip had spread the edges were sealed with vaseline. The preparations were examined under oil and about 2000 cells in each preparation were counted. Of the total, 2 kinds were qualified as to staining characteristics and per cent of the total. The cytoplasm of one kind is relatively completely full of stained intermeshed threads that encircle the nucleus. The second contains patches of the thread-like material and may contain stained granules. By usual definition both kinds of cells are reticulocytes and for present convenience these cells will be designated respectively, reticulocytes I and II. *Leucocyte* counts were made conventionally using a suitable diluting fluid. Plasma iron(7) and blood hemoglobin(8) were estimated colorimetrically. Hemin was prepared and purified classicly(9,10). Adult, male, *Pekin ducks* weighing 2.3-3.1 kg were used. They were watered and fed a commercial ration *ad libitum*. Blood was taken in heparin, small samples from a wing vein, large samples from the heart.

Experimental. Contribution by leucocytes to iron uptake. Since the level of leucocytes was expected to fall after irradiation, special test was made for their contribution to the uptake of iron. From centrifuged blood, preparations were made containing fewer or more leucocytes then the samples of blood from which the preparations were made. The level of leucocytes and uptake for such preparations and the blood from which they were made are given in Table I. The data indicate that leucocytes contribute little to the uptake. This is also implicit in the several reports(3-6) concerning uptake of iron.

Effect of whole-body x-irradiation on uptake of iron, leucocytes, plasma iron, hemoglobin, and reticulocytes. The animals, confined in a wire cage, were irradiated individually with X-rays, 250 KVP, 30 ma, 2.8 mm Cu HVL. The dose rate in air, with cage in place, and at a fixed point corresponding roughly to the center of the animal, was about 60 r per minute.

TABLE I. Uptake of Iron by Blood Cells.

Cell preparation	Leucocytes/ml of incubated mixture		Uptake	
	Cell preparation	Blood	Cell preparation	Blood
1	2400×10^5	100×10^5	.03	.21
2	13×10^5	81×10^5	.12	.16
3	36×10^5	72×10^5	.22	.23

Cell preparation 1: The buffy coat from 150 ml of centrifuged blood was suspended in equal vol of plasma. *Preparation 2:* Sample of blood centrifuged. The top fifth of the cells was discarded. The remainder was mixed with sufficient plasma to give the same hematocrit as the original blood. *Preparation 3:* The buffy coat and a minimum number of erythrocytes from centrifuged blood was discarded. The cells were mixed with sufficient plasma to give the same hematocrit as the original blood.

In the irradiated series, blood was collected for test before irradiation, either the day before or on the same day. The time of this sampling is considered Day 0. Additional samples were collected on Days 1-7 after the irradiation. In the control series, samples were taken during an eight-day period, the time of the first samples being considered Day 0. Except where plasma iron was assayed, no more than 5 samples of blood, making a total of 10 ml, were taken from any one animal. Where plasma iron was assayed, no more than 16 ml (about 5% of the blood volume) was taken. This allowed 4 measurements of plasma iron or fewer of iron plus other materials.

Because of the considerable individual variation in uptake, etc. (cf. legend of Fig. 1) and in order that each animal serve as its own control, all values of uptake, etc., were divided by the value obtained with the first sample of blood. The data are summarized in Fig. 1.

Following irradiation the uptake decreased by about one-half at Day 1 and reached a minimum at Day 2. The fall in leucocytes level was not marked until Day 2 and does not, therefore, account for the decrease in iron uptake. Again, it appears that the uptake is referrable to the erythrocytes.

The erythrocytes take up plasma iron as well as that added(4). An increase in the

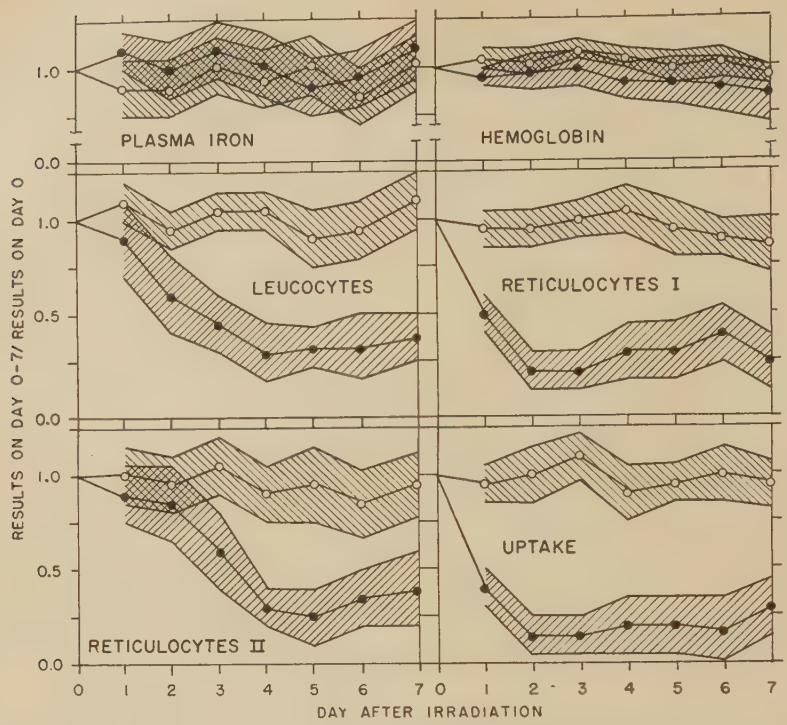

FIG. 1. Effect of whole-body x-irradiation on number of reticulocytes, number of leucocytes, hemoglobin concentration, plasma iron content, and uptake of iron by blood cells. Each value of uptake, etc., for any one animal was divided by the corresponding value obtained with the first sample of blood from that animal. The mean of the resulting relative values is given, the open circles representing controls and the solid circles the irradiated animals. The shaded area indicates twice the stand. dev. of the mean. Day 0 signifies a time before irradiation or, in the case of controls, the first of an 8-day period. For each test on Days 1-4 samples of blood from at least 5 animals were studied, for each test on Days 5-7 at least 3 samples were studied. The means of the actual results of the various assays and the corresponding stand. dev. for unirradiated animals were: Uptake, 0.20 ± 0.16; hemoglobin, 7.3 ± 0.5 μmoles/ml; reticulocytes I, $3.2 \pm 1.7\%$ of the total erythrocytes; reticulocytes II, $7.0 \pm 3.0\%$; leucocytes, $8.0 \pm 3.1 \times 10^3$/mm^3; plasma iron, 0.032 ± 0.011 μmoles/ml. The radiation dose was 600 r.

level of plasma iron would, therefore, decrease the uptake as measured. Also, as measured, the uptake would vary with the number of erythrocytes in the blood. However, there was no change in concentration of plasma iron attributable to the irradiation or, as indicated by the essentially constant level of hemoglobin, in numbers of erythrocytes. In the rat, in contrast to the present results in the duck, irradiation is followed by an increase in plasma iron(11).

The decrease in uptake is consequently referrable to change in particular erythrocytes. The level of reticulocytes II decreased after the irradiation, but marked change did not occur until after Day 2 when the uptake was at a minimum. The fall in reticulocytes I closely paralleled the fall in uptake. It appears, therefore, that these erythrocytes account for most of the iron removed from plasma by the blood cells.

It is worth pointing out that, in addition to the difference in morphology, indicated by staining, and in metabolism, indicated by the markedly greater iron uptake, the relatively early fall in reticulocytes I after irradiation indicates that these are younger than class II.

The similar degree of change in uptake and number of reticulocytes I make it unlikely that the metabolism of these cells was affected by the irradiation. However, the following additional tests for a change in metabolism were carried out: Plasma from control and irradiated animals was exchanged. Also, blood

was irradiated *in vitro*. Neither procedure affected the uptake. Large samples of blood, 100-150 ml, collected from 3 control animals and from 3 others at Day 1 after irradiation were incubated as usual, but for 12 hours, with radioactive iron and the uptake determined. Hemin was prepared from the washed cells and its activity determined. The fraction of the activity in the cells that was recovered as hemin was then calculated(3,4). The control values were 0.70, 0.80, and 0.82. The values for the irradiated animals were 0.75, 0.76 and 0.84. The irradiation, therefore, does not appear to affect the ability of the reticulocytes to incorporate iron into hemin.

Since no change in the metabolism of the circulating erythrocytes was evident, it seems that the decrease in uptake after irradiation results simply from a decrease in erythrocyte production, normal maturation of reticulocytes I and consequent loss of ability to take up iron.

Summary. Whole-body x-irradiation of the duck is followed by a decrease in the uptake of iron by erythrocytes *in vitro*. The decrease is attributable to a decrease in number of one kind of reticulocyte, which appears to be the youngest circulating erythrocyte. The level of these cells in blood falls because of a decrease in erythropoiesis and the usual maturation of these cells, which is accompanied by a loss in ability to remove iron from plasma.

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Serum Transaminase as a Measure of Myocardial Necrosis. (21705)

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Glutamic-oxaloacetic transaminase, an enzyme found widely distributed in body tissues, catalyzes the reversible transfer of an alpha-amino group from glutamic acid to oxaloacetic acid(1-3). Recently LaDue, Wroblewski and Karmen have reported on the occurrence of this enzyme in human serum and have observed elevated levels after myocardial infarction(4). The present report presents some observations on the properties of the enzyme in normal human serum and a preliminary evaluation of the serum concentrations as an index of myocardial necrosis. The method of assay has been adapted for clinical use to a more generally available, less expensive spectrophotometer.

Materials and methods. Transaminase activity was assayed by a modification of the

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method of Karmen, Wroblewski and LaDue (5). In this method the transaminase reaction (I) is coupled with the malic dehydrogenase reaction (II).

I Aspartate + α -ketoglutarate $\rightleftharpoons$ glutamate + oxaloacetate

II Oxaloacetate + DPNH + H^+ $\rightleftharpoons$ malate + DPN^+

At appropriate levels of substrates, enzyme and reduced coenzyme the rate of disappearance of reduced diphosphopyridine nucleotide (DPNH) is proportional to transaminase concentration. *Malic dehydrogenase* used in most of these studies was isolated in partially purified form from *Escherichia coli*. From crude extracts of the organism enzyme of high activity and essentially free of transaminase can be prepared in good yield with a three step procedure(6). The enzyme can be stored in the frozen state for as long as 6 months without appreciable loss of activity. Recently we have used a commercial preparation isolated from pig heart by the more laborious method of Straub(7). No important differences in the behavior of the two preparations in the assay was noted. 0.5 ml 0.2 M aspartate, 0.2 ml DPNH (1 mg/ml), 1.9 ml 0.1 M phosphate buffer pH 7.4 and 5 microliters of the malic dehydrogenase preparation (15 micrograms of protein) are mixed in a Beckman cuvette. The control cell is identical except that the DPNH is replaced by phosphate buffer. Optical density at 340 mμ is followed in a Beckman DU Spectrophotometer as a measure of DPNH concentration. When 0.2 ml serum, normal or pathological, is added there is first a rapid, then a much slower disappearance of DPNH which continues in different sera for as little as 5 minutes or as long as 30 minutes. While this intrinsic dehydrogenase activity is usually exhausted in the 10 minute interval recommended by Karmen *et al.*(5), in order to obtain accurate values in sera of low activity we find it necessary to delay 30 minutes before proceeding with the assay. No decrease in serum transaminase activity is observed even when cuvettes are allowed to stand 1½ hours at room temperature at this stage in the assay procedure. Extra DPNH was added to the reaction mixture after 30 minutes and was found to be stable except for the same minimal rate of disappearance observed in the enzyme blank (Fig. 1), indicating that the intrinsic DPNH-oxidizing action is truly exhausted. At 30 minutes, then, 0.2 ml 0.1 M alphaketoglutarate is added. There follows a rapid decline in absorption at 340 mμ, reflecting the initiation of the transaminase reaction. We have observed that the *reaction rate* during the first 2-3 minutes is in some cases less than maximal. For this reason, instead of using the change in optical density in the first 10 minutes after addition of the ketoglutarate (5), we have followed the optical density at 1-2 minute intervals and made our calculations from the linear portion of the curve. When optical density falls below 0.200 the curve often departs from linearity. The observed linear rate is corrected for the small enzyme blank. Results are expressed in units per ml of serum. One unit is defined as that amount of transaminase causing optical density at 340 mμ to change at the rate of 0.001 per minute per cm of light path under the conditions of the assay. Replicate assays for the same serum agreed to within 5%. The *assay* can be more conveniently although not quite so accurately carried out using the Bausch and Lomb Spectronic 20 Colorimeter. The manufacturer is able to calibrate this instru-

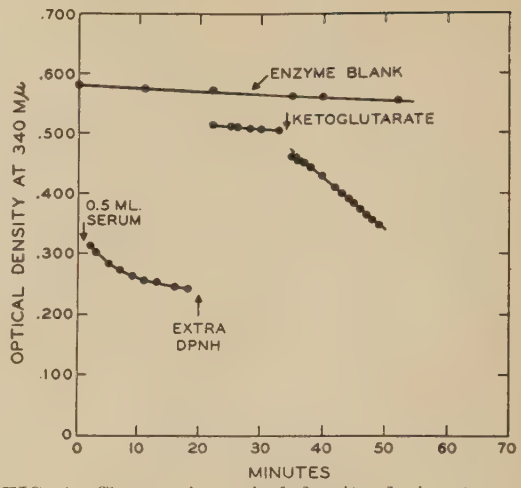

FIG. 1. Changes in optical density during transaminase assay without (upper curve) and with serum (lower curve). Extra DPNH was added in this case to demonstrate that the endogenous activity of serum in oxidizing DPNH was completely exhausted at 20 min.

ment at 340 mμ even though the nominal lower limit of its range is at 350 mμ.† When cuvettes with a 1 cm light path are used the procedure is the same as that described above. When cuvettes of different size are used or when matched test tubes are used it is suggested that results be expressed in optical density units *per cm of light path* to conform to the unit used by Karmen *et al.*(5). For example, using matched ½ inch round test tubes we found the 340 mμ readings in the Spectronic 20 to be about 15% lower than in the 1 cm square Beckman cuvette. When the observed rates of optical density decrease were appropriately corrected on this basis results obtained for the same sera on the two instruments agreed within 10%.

Occurrence and properties of the enzyme. The enzyme was present in all sera tested. In 20 normal adults the concentrations ranged from 10 up to 33 units per ml. The levels in normal individuals were quite constant from day to day and did not appear to be altered appreciably by meals. *Normal urine* contains less than 1 unit per ml. An attempt was made to concentrate any enzyme present in the urine by ammonium sulfate precipitation but the excessive 340 mμ absorption in the concentrate interfered with the assay. As it occurs in serum the enzyme is remarkably stable, full activity being maintained in some samples stored at refrigerator temperatures for up to 3 weeks. The enzyme is already fully activated in serum since the addition of 0.3 mg/ml of its coenzyme, pyridoxal phosphate, caused no change in the assay(8,9). Addition of water to produce hemolysis released enough enzyme to increase the assay per ml almost 8-fold. Care was exercised during the handling of blood samples to avoid hemolysis.

Results in myocardial infarction. Of 24 consecutive cases studied in which the electrocardiogram showed unequivocal evidence of myocardial necrosis all but 2 showed an elevation of the serum transaminase above 40 units per ml when samples were obtained within 48 hours after the onset of pain.

† The modified instrument used was made available through the courtesy of J. Westcott Wright of Bausch and Lomb Optical Co.

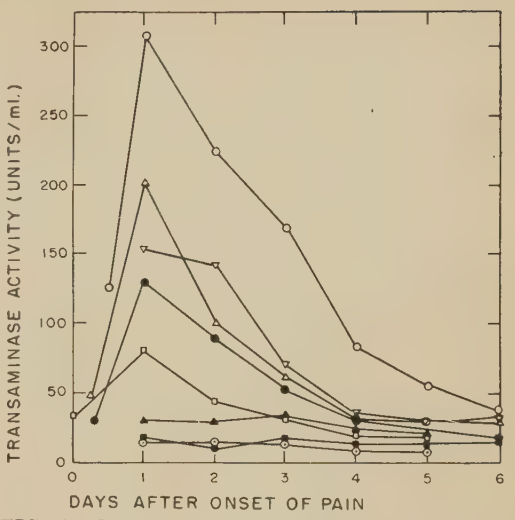

FIG. 2. Serum transaminase levels in 5 typical cases of myocardial infarction (upper curves) and in 3 cases of angina pectoris (lower curves).

In Fig. 2 are presented the results of daily serum transaminase assays in several typical cases from this group. Typically the serum levels rose sharply to a peak within 36 hours after the onset of pain and then fell somewhat less rapidly, reaching normal values within 4 to 6 days. The peak values observed ranged from 54 to 308 units per ml with a mean of 154 units per ml.

In contrast, patients with the transient myocardial ischemia of angina pectoris without myocardial necrosis showed no elevation of transaminase. The three curves at the bottom of Fig. 2 show that in such cases one observes only the normal minimal day to day fluctuations.

In addition to glutamic-oxaloacetic transaminase two other enzymes were assayed in sera from some of the patients in this study. These were adenosine triphosphatase(10) and lipoprotein lipase(11). No correlation was found between the levels of these enzymes and the occurrence of myocardial necrosis.

Discussion. The very consistent rise in serum transaminase levels after myocardial infarction is presumably due to the release of this enzyme from damaged heart muscle, in which it is present at high concentrations(2). The rapid rate of disappearance, despite the demonstrated stability of the enzyme in

serum, indicates the existence of a very effective mechanism for its removal from the blood. The observed curve is then the resultant of the rate of release from the heart and the rate of removal by an as yet unspecified mechanism.

Since the enzyme is very widely distributed it might be anticipated that damage to other tissues than heart muscle might also raise the levels in serum. We have observed slight elevations after abdominal surgery. Striking elevations (over 100 units per ml) were found in cases of diffuse liver disease (portal cirrhosis and biliary cirrhosis). Preliminary studies in collaboration with Dr. Andrew G. Morrow indicate that experimental infarction of the bowel in dogs can cause significant elevations. On the other hand normal levels were observed in uncomplicated cardiac failure, acute pneumothorax, pericarditis, nephrosis with marked edema, and in some cases of pulmonary infarction.

Thus while the test is not absolutely specific for myocardial necrosis the excellent correlation with that condition suggests the possible usefulness of serum transaminase assays in diagnosis.

Summary. Myocardial necrosis in humans was accompanied in over 90% of the cases studied by elevation of the serum glutamic-oxaloacetic transaminase. Serum adenosine triphosphatase and serum lipoprotein lipase were not elevated. Within 36 hours after myocardial infarction the transaminase levels rose to 2-10 times normal and usually returned to normal within 5 days. The myocardial ischemia of angina pectoris was not accompanied by any elevation of transaminase levels.

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Lack of Lasting Protective Effect of Isoniazid in *M. lepraemurium* Infection of Rats. (21706)

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It has been well established that isoniazid exerts a pronounced prophylactic effect against the *M. lepraemurium* infection of mice (1-4) and rats(4-8). This marked activity was observed regardless of whether the route of infection was intravenous(1), intracorneal (2), intraperitoneal(3,4,8), subcutaneous (5,6), or intramuscular(7) and regardless of the time when therapy was instituted, whether it was immediately(5-8) or 17 to 28 days (4,6,7) after infection. In all these experiments the animals remained on therapy until they were sacrificed and examined for the presence of bacilli or lepromas. No conclusion could, however, be drawn as to whether or not isoniazid exerts a lasting effect upon *M. lepraemurium,* a fact which has been observed with *M. tuberculosis*(9). The present experiments were, therefore, undertaken in which animals received isoniazid for periods varying from 4 to 22 weeks and were kept under observation for as long as 46 weeks after discontinuation of treatment.

Materials and methods. The Hawaiian

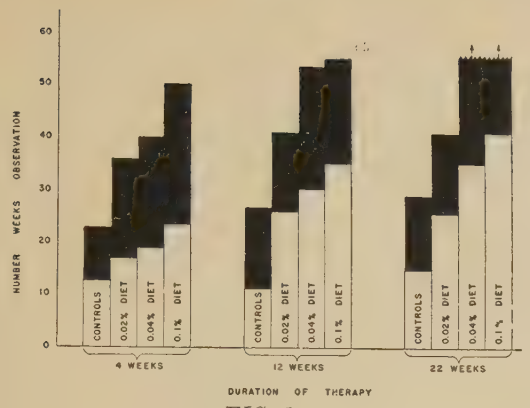

FIG. 1.

strain of *M. lepraemurium*, obtained through the courtesy of Dr. John Hanks, was used in all experiments. A 10^{-1} by weight suspension in water of a rat leproma was prepared according to the technic described previously (10). White albino rats [Wiersing Strain (10)] were infected subcutaneously in the right axillary region with 0.5 ml of this suspension. The animals were immediately placed on a medicated diet containing either 0.1%, 0.04%, 0.02%, 0.01% or 0.004% isoniazid. Groups of 10 rats for each dose of isoniazid used were fed the medicated diet for either 4, 12 or 22 weeks at which time treatment was discontinued and the animals observed for a total maximum period of 56 weeks post infection. The controls consisted of a group of rats infected with the live organisms and a group infected with the same number of autoclaved bacilli.

Results. The data obtained from the experiments in which rats were fed the medicated diets containing 0.1%, 0.04% or 0.02% isoniazid for either 4, 12 or 22 weeks are graphically depicted in Fig. 1. Not included in this chart are the results obtained from those animals receiving 0.01% and 0.004% isoniazid since they did not differ from the controls inoculated with live bacilli.

In Fig. 1, the upper level of the clear portion of the bar indicates the time at which measurable tumors were first observed in the rats. The upper level of the shaded portion of the bar indicates that time at which 90% to 100% of the rats had succumbed. Fig. 1 undoubtedly shows that as compared with the controls there was an extension of both the time at which the first lepromas appeared and the final survival time at all dosage levels in all treatment periods. There was a direct correlation between the time of development of leproma and the extension of the survival time with the increase of dose or the extension of the treatment period.

In only 2 groups of rats, namely those receiving a diet containing 0.1% and 0.04% isoniazid for 22 weeks did the majority of animals survive the 56-week observation period. These rats had, however, fully grown lepromas.

As might be expected, those animals receiving autoclaved bacilli showed no signs of tumor during the entire observation period.

Discussion. The present experiments have shown that in rats infected with *M. lepraemurium* an increase in either dose or length of treatment with isoniazid results in a delay of both the first appearance of lepromas and death of the animals. Tumors did, however, develop in all animals even in the group treated with 0.1% medicated diet for as long as 22 weeks although the animals in this group as well as those receiving 0.04% diet were still surviving at the end of the 56-week observation period. The presence of fully developed tumors allows the conclusion that they probably would have succumbed if observed for a sufficient period of time. It may, therefore, be concluded that under the experimental conditions employed no lasting protective effect was observed. Isoniazid suppresses but does not totally eradicate the bacilli. The toxicity of isoniazid, of course, limits the question of whether considerably higher doses could eradicate an infection due to *M. lepraemurium.*

Summary. 1. Isoniazid delays the development of the leproma and extends the survival time of white rats infected with *M. lepraemurium.* 2. Both phenomena are directly proportional to the increase of dose as well as to the extension of the duration of therapy with isoniazid. 3. Viability of the bacilli is affected by therapy but under the experimental conditions used the organisms are not completely eradicated.

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Metabolism of 5-Hydroxytryptamine (Serotonin) by Monoamine Oxidase.* (21707)

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There is evidence that 5-hydroxytryptamine (5HTA) is metabolized by oxidative deamination(1). The product of this reaction would be the aldehyde which is then further oxidized to 5-hydroxy-indoleacetic acid (5HIAA). Five-HIAA occurs in normal urine and the amount increases following the administration of 5HTA(2). There is as yet insufficient evidence as to whether monoamine oxidase(3) is the tissue catalyst involved in the deamination of 5HTA and whether this enzyme is mainly responsible for the *in vivo* disposition of the compound.

The experiments reported here were designed to elucidate the role of monoamine oxidase in the metabolism of 5HTA.

Methods and materials. Male rabbits(1.3-2.0 kg) and rats (250-300 g) were sacrificed by decapitation. Brain, liver and kidney were removed immediately, blotted to remove excess blood, chilled and homogenized in 2 volumes of water. Similar homogenates were prepared of liver and kidney removed from a dog during nembutal anesthesia. Mitochondria were isolated from rat liver homogenate by the method of Hogeboom *et al.*(4). Five-HTA as the creatinine sulfate† and tyramine as the monohydrochloride were prepared as 0.012 M solutions in 0.01 N hydrochloric acid. Incubations were carried out in 20-ml beakers in air for one hour on a Dubnoff metabolic shaking incubator at 37°C. Incubation mixtures contained 1 ml of substrate (5HTA or tyramine), 1 ml of homogenate, 0.3 ml of 0.5 M phosphate buffer pH 6.9, made to a final volume of 3 ml with water. Monoamine oxidase was inhibited by the addition of 0.2 ml of l-isonicotinyl 2-isopropyl hydrazide (IIH)‡ to give a final concentration of 4×10^{-4} or 4×10^{-3} M. A 15-minute preincubation with inhibitor, prior to the addition of substrate, was required to give optimal inhibition. Other experiments included controls which lacked either enzyme or substrate. Enzyme activity was measured by the disappearance of substrate. Five-HTA was assayed colorimetrically after extraction from the incubation mixture by the method described by Udenfriend *et al.*(5). Tyramine was extracted in the same manner as 5HTA but was assayed by the procedure of Udenfriend and Cooper(6).

* Studies on prolongation of 5HTA effect *in vivo* by IIH were carried out by Mr. Herbert Weissbach.

† 5HTA was supplied by Abbott and the Upjohn Laboratories as the creatinine sulfate complex.

‡ IIH (Marsilid) was obtained through the courtesy of Hoffmann-La Roche, Inc.

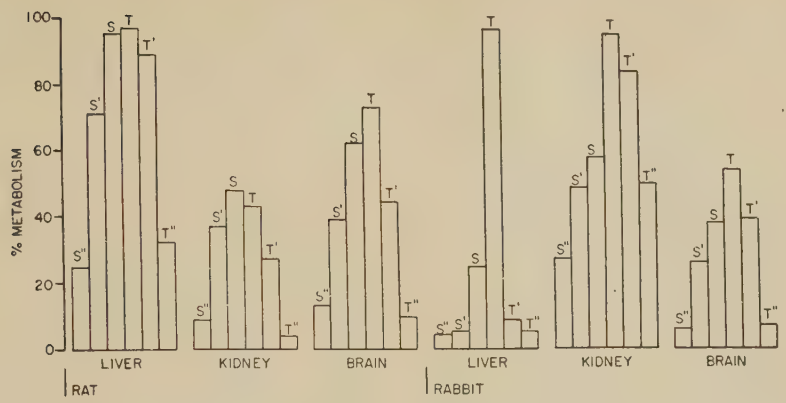

FIG. 1. 5-hydroxytryptamine and tyramine metabolism in rat and rabbit tissue homogenates and inhibition by 1-isonicotinyl-2-isopropylhydrazide (IIH). All incubations were for 1 hr as described in the text. S = control 5HTA, T = control tyramine, S′ = 5HTA plus 4×10^{-4} M IIH, T′ = tyramine plus 4×10^{-4} M IIH, S″ = 5HTA plus 4×10^{-3} M IIH, T″ = tyramine plus 4×10^{-3} M IIH.

Results. Comparison of in vitro metabolism of 5HTA with that of known substrate of monoamine oxidase, tyramine: The metabolism of 5HTA and tyramine by brain, liver and kidney homogenates of rat and rabbit is shown in Fig. 1. Inhibition of 5HTA and tyramine metabolism by IIH is also shown. The amounts of 5HTA and tyramine metabolized by each tissue were similar, with the single exception of rabbit liver. More significant is the fact that IIH was an excellent inhibitor of both 5HTA and tyramine metabolism in all tissues, including rabbit liver. Mitochondria prepared from rat liver homogenates were also shown to metabolize both amines and the ratios of tyramine to 5HTA metabolism were 2.0 to 1.5 in the mitochondria and in the homogenate from which they were prepared. Furthermore, IIH was also an effective inhibitor of the mitochondrial activity. Neither tyramine(7) nor 5HTA is metabolized by liver microsomes.

Effect of in vivo administration of IIH on 5HTA and tyramine metabolism: a male dog weighing 13.7 kg was anesthetized with nembutal. IIH (35 mg/kg) was administered intravenously following removal of one kidney and a portion of the liver for control measurements. Ninety minutes later the other kidney and additional samples of liver were obtained. Portions of the control tissues and of the tissues removed after IIH injection were incubated with 5HTA and tyramine. As shown in Table I marked inhibition of the metabolism of both amines was produced by the previous *in vivo* administration of IIH.

Potentiation of 5HTA effects in vivo by IIH: Male rats (ca 300 g) were given 3 mg of 5HTA intraperitoneally. Six rats were pretreated with IIH in a dose (25 mg intraperitoneally) which in itself produced no apparent effect. Thirty minutes after IIH injection, each animal was given 5HTA. In every case, the following effects, which lasted at least three hours, were observed: a reddish-blue discoloration of the extremities and tail, decreased temperature (to touch) of the skin, incoordination of the hind legs and marked tranquillization. In control animals the effects of 5HTA were not readily discernible. Of 6 control animals, only one showed definite signs which lasted for 15 to 20 minutes and were identical to those in the IIH-5HTA treated group.

TABLE I. Effect of *In Vivo* Administration of 1-Isonicotinyl-2-Isopropyl-Hydrazide (IIH)* on 5-Hydroxytryptamine (5HTA) and Tyramine Metabolism in Dog Tissue Homogenates.

	% 5HTA metabolism†		% tyramine metabolism†	
Tissue	Before IIH	90 min. after IIH	Before IIH	90 min. after IIH
Liver	52	8	99	52
Kidney	45	25	99	45

* 35 mg/kg intrav.
† During 1-hr incubation.

Discussion. Blaschko(1,8), Blaschko and Philpot(9), Freyburger, *et al.*(10) and Govier, *et al.*(11) have implicated the monoamine oxidase system as a pathway of 5HTA metabolism. This conclusion has been based on experiments showing similar rates of O_2 uptake by tissue homogenates incubated with 5HTA and with tyramine, the latter a known substrate of monoamine oxidase. This evidence is suggestive, but by no means conclusive.

The disappearance of these 2 amines in tissue homogenates of three animal species confirms their general similarity of metabolism. The extremely slow rate of 5HTA metabolism in rabbit liver, a tissue which metabolizes tyramine rapidly, is unexpected, but the finding is similar to the results of Schayer, *et al.*(12) with tryptamine. The consistent inhibition of 5HTA and tyramine metabolism by IIH *in vivo* and *in vitro* implicates the monoamine oxidase mechanism more specifically. The demonstration of metabolic activity for 5HTA in mitochondria where monoamine oxidase has been shown to occur by Hawkins(13), and the ability of IIH to inhibit the mitochondria activity is further evidence of monoamine oxidase action. Finally, the marked *in vivo* prolongation of 5HTA effects by IIH provides almost conclusive evidence that monoamine oxidase plays a major role in the metabolism of 5HTA in animals.

It has been shown that 5HTA is rapidly metabolized following injection into the dog and rat and is excreted as 5HIAA(2). The rapidity with which 5HTA is metabolized, apparently by monoamine oxidase, suggests that this enzyme may be directly involved in limiting the physiologic action of 5HTA, just as cholinesterase controls the action of acetylcholine. The other physiologically active monoamines, epinephrine and norepinephrine, are such poor substrates for this enzyme that it has long been felt that monoamine oxidase could not be involved in adrenergic function. Recent experiments by Griesemer *et al.*(14) and by Küelle and Volk(15) support the view that monoamine oxidase is not related to adrenergic function. It is possible that the only physiologic action of monoamine oxidase is on 5HTA.

Summary. On the basis of: (1) Comparable rates of 5HTA and tyramine metabolism in tissues of 3 different animals; (2) comparable inhibition of metabolism of both amines by IIH, a potent monoamine oxidase inhibitor; (3) a demonstration that 5HTA is metabolized by the mitochondrial portion of the liver cell; and (4) a prolongation of the effects of 5HTA *in vivo* following pretreatment with IIH, it is concluded that monoamine oxidase is the major pathway of 5HTA metabolism in animals.

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Effect of Roentgen Irradiation and Other Types of Injurious Agents on Capillaries.* (21708)

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When animals are injured in various ways the capillary circulation suffers. Yet very little effort has been made to study the capillaries damaged as a result of such injury. Capillary fragility, that is to say the inability of capillaries to withstand mechanical forces acting upon them, is said to increase during x-ray sickness and other types of shock, but it has never been measured directly. A technic has been developed in this laboratory to permit the measurement of breaking strength of living microscopic structures; this method has been extended to the direct measurement of capillary fragility. The results of investigations using this method on the capillaries of injured animals are here reported.

Methods. Experiments were performed on the capillaries of the mesentery of the leopard frog, *Rana pipiens*. The animals were equilibrated with room temperature (18-22°C) for one week prior to experimentation. The capillary preparation was made as follows: A frog was pithed, placed on a glass plate, and a small lateral abdominal incision was made. A loop of intestine was exposed and spread on the glass plate. The mesentery was kept moist with Ringer solution, and the whole preparation was placed on the movable stage of a compound binocular research microscope. Determinations of *capillary fragility* were made by the microinjection method of Rieser(1) for the determination of resistance of cell membranes to internal pressure. In the present experiments, a glass micro-pipette (diameter of opening at the tip 2-5 μ) was inserted into a suitable capillary by means of a micromanipulator. A pressure gauge, reading directly in millimeters of mercury, registered the pressure at the tip of the micropipette when fluid was injected into the capillary. Pressure was applied until the capillary ruptures; the reading on the gauge at this point represented the breaking strength of the capillary wall. This was taken as the index of capillary fragility. Some consideration must be given to the question whether the pressure recorded by the gauge represents the pressure which acts within the capillary during the injection. The flow of fluid through the micropipette might reduce the pressure existing at its orifice. This difficulty was resolved by applying the pressure gradually. In order to accomplish this, the plunger of the microinjection syringe was rotated within the cylinder; at each half-revolution during its downward travel the plunger was stopped for an instant. This provided for the existence of true hydrostatic conditions; only in this manner will the recorded pressure correspond to the actual pressure at the micropipette orifice. There remains, however, the consideration of the curved surface of the fluid inside the micropipette. In forcing the fluid through its orifice, the greater pressure is on the concave surface of the fluid inside. If the radius of curvature of this surface exceeds a maximum value, an additional amount of pressure must be applied, and flow would result, thereby upsetting hydrostatic conditions. A correction for this was worked out and is so small as to be negligible. Another difficulty which could interfere is that an adsorbed surface layer on the walls of the micropipette or attraction of the surface molecules of the walls upon the inclosed fluid could cause a deviation from the laws of viscous flow. However, Bulkley(2), working with various liquids in glass capillaries of 5.95 μ internal radius, found that there was no such deviation. With these difficulties solved, it was felt that the method of measuring pressure acting of the capillary wall from within was satisfactory. As a matter of fact, Landis(3) measured the capillary pressure in the frog mesentery in essentially the same manner. *Roentgen irradiation* of frogs

* This investigation was supported by a research grant from the U. S. Atomic Energy Commission, administered by L. V. Heilbrunn.

TABLE I. Breaking Strengths of Mesenteric Capillaries of Normal Frogs and Frogs with Irradiation and Other Types of Injury.

	Controls	3600 r	7200 r	Heparin	Trypsin	Peptone
No. of animals	16	4	8	10	4	5
Total No. of observations	19	4	15	15	9	11
Mean pressure, mm Hg	87 ± 36*	34 ± 16	29 ± 10	37 ± 17	37 ± 16	21 ± 14

* Stand. dev.

was performed using an apparatus of the American Oncologic Hospital. The author wishes to express his gratitude to Messrs. J. L. Weatherwax and L. Stanton for kindly making their x-ray facilities available to him. The conditions of irradiation were 140 kilovolts, 5 milliamperes, 3 mm half-value layer filtration, and 20 cm target distance. The output of the machine was 150 r per minute. Subsequent to irradiation, frogs were kept in individual glass containers at room temperature until they showed the first visible signs of irradiation sickness (discharge of mucus, lack of motor coordination, depression) before being subjected to experiments. The effect of *heparin* injection on capillary fragility was studied by injecting heparin directly into the capillaries. A 0.1% solution of heparin (Upjohn, 136 Toronto units per mg) was used. *Trypsin-treated* and peptone-treated frogs were kindly supplied by Dr. V. Weimar. Trypsin (Worthington's crystalline lyophilized salt-free) in Ringer was administered via the ventral lymph sac in a concentration of 0.01 mg per gram of body weight 2 hours before measurements of capillary fragility were made. *Peptone* (Difco Bacto Protone No. B125) in Ringer was administered via the ventral lymph sac in a concentration of 10 mg per gram of body weight 2 to 5 hours before fragility measurements were begun.

Results. The results are summarized in Table I. The average breaking pressure of mesenteric capillaries in the normal frog (capillaries injected with Ringer solution) is 87 mm of mercury. Significantly lower pressures were obtained after roentgen irradiation, heparin injection, trypsin and peptone shock. The fragility measurements were made following a suitable time interval after the experimental treatment, *i.e.*, at or near the height of the damage produced by the injurious agent. Such frogs frequently presented, in addition to an altered external appearance (motor depression, discharge of mucus), evidence of internal damage such as petechiae in the muscles and intestine, hyperemia, hemorrhage, and liver color changes. These changes and other aspects of the injury are discussed in detail by Weimar(4). In the frogs studied, the injury was relatively slight; capillary fragility measurements could not be made in severely injured animals with pronounced hemorrhages, collapsed or static capillaries. Such capillaries could not be successfully entered by a micropipette, the attempted entry causing local disintegration of the capillary wall. Thus the figures in Table I probably represent only minimum effects on capillary fragility.

Discussion. According to Moon *et al.*(5) there is a close similarity between radiation sickness and shock due to other causes; thus irradiation can be used as a means of producing shock injury. Not much has been done in the way of a direct study of capillary damage during x-ray and other types of shock. One frequently finds statements in the literature to the effect that generalized capillary damage occurs, and that capillary permeability is increased in shock. There exists hardly any direct information concerning capillary fragility. Griffith *et al.*(6) found an increased number of petechiae in the rat's peritoneum following alpha irradiation, suggesting increased fragility of the capillaries. Their method, however, was only qualitative. In the present investigation the effect of roentgen irradiation was to produce a marked increase in capillary fragility as evidenced by the strikingly lower breaking pressures of the capillaries. No conclusive evidence exists in order to account for the increased capillary fragility during x-ray and other injury found here; this is to be regarded as a preliminary report. Suggestive evidence nevertheless ex-

ists. The data show that microinjection of heparin into the capillaries also lowers their breaking strength. This is in line with Zweifach's observation(7) that heparin, when applied locally to capillaries, produces petechial hemorrhages through the capillary walls. The possibility exists that irradiation of the frogs releases heparin into the circulation, and this heparin had the effect of damaging the capillary walls. Evidence for such an increased circulating anticoagulant following total body irradiation was found by Allen and Jacobson (8). The anticoagulant was found to be heparin. Smith, Svihla and Patt(9), studying the circulation in the bat's wing, favor the view that x-irradiation either damages the capillary wall directly or by the formation of a humoral agent. This agent might conceivably be heparin.

If the lowered capillary breaking pressures (increased capillary fragility) in x-ray shock can at least in part be explained by the liberation of heparin, then it is likewise possible to account for the lowered capillary breaking pressures found with trypsin and peptone shock. In trypsin shock, according to Rocha e Silva and Dragstedt(10), the trypsin is known to liberate heparin. Similarly, heparin is released in peptone shock(11,12). There thus appears to be supporting evidence for the suggestion that in the cases studied, roentgen irradiation, trypsin and peptone shock, a release of heparin is responsible for the increased capillary fragility.

Summary. By means of a microinjection method it was possible to measure the fragility of mesenteric capillaries of the frog. Roentgen irradiation, heparin injection, trypsin and peptone shock increase the fragility of the capillaries. The increased capillary fragility appears to be a consequence of a liberation of heparin into the circulation of the animals, due to the irradiation or shock treatments.

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Bio-Assay for Anti-Inflammatory Substances. (21709)

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The ever increasing use of ACTH, cortisone, and related steroids as therapeutic agents for the reduction and treatment of inflammatory diseases has greatly stimulated efforts to elucidate the fundamental mechanism of inflammation(1,2,3). One of the serious difficulties in such work and in the evaluation of antiinflammatory substances has been the lack of a reliable bio-assay.

The search for a method that could be applied to normal animals on various nutritional regimes eliminated from consideration the histologic technics of Dougherty(4) and

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TABLE I. Swelling Index (S.I.) of Guinea Pig Ankle Joint following Intra-Articular Injection of 0.1 ml of Rabbit Anti-Guinea Pig Serum.

Antiserum	Mean wt (g)	Saline pre-treated 1½ hr, control (S.I.)	Mean wt (g)	Cortisone, pre-treated 1½ hr			
				50 mg/kg	25 mg/kg	10 mg/kg	5 mg/kg
				(S.I.)			
6	297	4.3 ± .52*	323				4.4 ± .27
	313	5.1 ± .51	292			4.5 ± .29	
	280	3.5 ± .32	290		1.9 ± .20		
	256	4.6 ± .65	250		1.9 ± .22		
	352	5.1 ± .45	327		3.7 ± .62		
	312	5.2 ± .50	302	2.8 ± .28			
4	317	5.1 ± .41	313				4.5 ± .46
	295	4.9 ± .60	319			4.6 ± .52	
	264	3.6 ± .18	266			3.3 ± .31	
	307	4.7 ± .48	305			3.9 ± .31	
	272	4.7 ± .28	308		3.0 ± .27		
	313	4.7 ± .37	303		2.7 ± .44		
	276	4.9 ± .27	287		2.2 ± .54		
Avg young animals	296	4.6	299	2.8†	2.6†	4.1	4.5
Avg adult animals	433	5.6	413		5.7		

* Mean and stand. error of mean, 6 animals/group.
† $P > .01$ when compared to control groups.

Gross(5), and methods using adrenalectomized or castrated animals such as the thymus involution technic of Dorfman(6), the eosinophil method of Speirs(7) and various stress tests. Numerous existing technics for the production of sterile inflammation in normal animals were tested, including the passive Arthus joint swelling method of Ungar *et al.*(8), the intradermal Arthus reaction of Humphrey(9), the granuloma pouch method of Selye(10), the serum diphenylamine method of Coburn and Haninger(11), and a number of simply-induced inflammations using graded doses of crystalline and crude egg albumin, dextran, compound 48-80, polyvinyl pyrrolidone, histamine, etc.; but all were rejected because of wide biological variation or lack of response to known protective agents.

Experimental. Following a suggestion by Dr. Saul Malkiel, Northwestern Medical School, rabbit anti-guinea pig serum was prepared (by a 3-week series of tri-weekly intravenous injections of pooled guinea pig serum into adult rabbits) that gave a severe reversed-Arthus reaction when injected intradermally into normal guinea pigs. When diluted 1:1 with physiological saline and injected (0.1 ml) into the ankle joint of young (300 g in weight) guinea pigs, this antiserum produced consistent inflammation and edema for measurement of the swelling index with a constant pressure micrometer caliper with ratchet stop, as outlined by Ungar *et al.*(8). Furthermore the expected edema could be consistently reduced about 50% by prior treatment of the animals with cortisone acetate (25 mg per kilo body weight, intraperitoneally, 1½ to 2 hours before the test). As shown by the data in Table I, lesser amounts of cortisone afforded decreased protection.

Discussion. A number of variables were found to influence the validity of the procedure, especially the size or maturity of the guinea pig, the method of measuring the joint edema, the selection and care of the animals, the method of intra-articular injection, and the relative amounts of antibody and of cortisone injected. Normal, healthy guinea pigs weighing about 300 g reacted consistently to the antiserum, while those weighing less than 250 g or more than 350 g were observed to react minimally and maximally, respectively (Table I). In determining the swelling index, normal rabbit serum diluted 1:1 with physiological saline was injected (0.1 ml) into the control ankle joint opposite to the one receiving the rabbit anti-guinea pig serum; and the differences in swelling between the 2

ankles, measured one hour after injection and each half hour thereafter for a 5-hour period, were totaled. Practice is necessary in the intra-articular injection to avoid traumatic injury, and in the transverse measurement of the joint to obtain consistent readings without compression of the edematous tissue. The ratchet stop spring supplied in the caliper was weakened considerably to avoid compression. It was necessary to use groups of 6 guinea pigs in each test to reduce the statistical variance, and to include a control (saline pre-injected) group from each new lot of guinea pigs when determining the anti-inflammatory potency of an unknown. No evidence of a sex difference in response could be observed when animals weighing 300 g were used.

This procedure has been applied with success to the determination of the relative anti-inflammatory effects of cortisone acetate, cortisone alcohol, hydrocortisone, salicylates, and dietary supplements of egg yolk phospholipid fractions.

Summary. A bio-assay for anti-inflammatory substances is presented. It is based on the reduction of swelling in the joints of guinea pigs which had been subjected to a severe reverse Arthus reaction.

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Synthesis of Lipids in Cell-Free Extracts of Yeast.* (21710)

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Several groups of investigators have recently described cell-free systems, derived from mammalian sources, capable of incorporating acetate into complex lipids such as cholesterol and high molecular weight fatty acids(1-4). No report of a similar microbial system has yet appeared.

In this laboratory incorporation of labeled acetate into fatty acids and non-saponifiable lipids has been obtained with cell-free homogenates. *Saccharomyces cerevisiae,* strain LK2G12, when grown under anaerobic conditions and subsequently aerated in resting cellular suspensions, forms large quantities of ergosterol and other lipids(5). If the cells are harvested during the period of aeration, washed, and then ruptured by means of a Mickle disintegrator, active preparations result.

Methods. The yeast cells were broken under an atmosphere of nitrogen in a solution containing 0.9% KCl and 0.02 M phosphate buffer (pH 7.2). After treatment in the Mickle apparatus, whole cells and cell walls were removed by two successive 10-minute centrifugations at 3600 rpm in an International centrifuge, Model PR-1. At this time careful observation by ordinary and phase contrast microscopy revealed no whole cells; only a dense sea of particles was seen. This homogenate was then incubated in air at 30°C in Warburg vessels containing carboxyl-

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TABLE I. Incorporation of Acetate into Fatty Acids (FA) and Non-Saponifiable Lipids (NSF) by Homogenates of Yeast, in 5 Experiments.

Treatment of cells	Acetate added, μmoles	μatoms acetate incorporated* FA	NSF
Standard†	3.2	3.17	2.74
"	3.2	2.00	2.57
Standard	3.2	.82	3.17
"	13.2	.11	10.00
Disintegrated in water	3.2	.00	.20
Standard	3.2	3.58	6.23
Disintegrated 15 min.	3.2	7.80	13.00
" 20 "	3.2	1.04	5.80
Standard	1.6	1.70	1.80
"	3.2	1.58	5.07
"	6.4	1.84	1.86
Standard	3.2	.55	1.75
Disintegrated 15 min.	3.2	2.16	6.34
" 30 "	3.2	1.65	2.55
" 60 "	3.2	1.62	2.26

* Calculated after Bucher(8): μg atoms of acetate carboxyl carbon incorporated into FA or NSF /g dry wt of homogenate/hr.

† In the standard treatment, 5 g yeast paste is disintegrated in the Mickle apparatus for 5 min. in 4 ml KCl-phosphate solution, containing 6 g glass beads as described in the text. 1 ml of the homogenate, freed of intact cells, is used/flask, together with 0.1 ml of an acetate-ATP solution containing 2 μmoles ATP as shown in table. The center well of each vessel contained 0.2 ml 20% KOH. Other conditions given in the text.

labeled acetate (5.27×10^5 cpm per vessel) and ATP. After 4 hours, metabolic activity was stopped by the addition of 0.5 ml saturated KOH to each vessel. The flask contents were then removed and combined with approximately 200 mg (dry weight) of unlabeled intact yeast cells to serve as carrier during the subsequent extraction procedures (6). The lipids were plated directly on copper discs and counted with a Tracerlab gas-flow counter.

Results. The results of a series of experiments are given in Table I. Several lines of evidence, in addition to that obtained by microscopic examination, confirm the contention that the results given in the accompanying table are those of a cell-free system. The addition of labeled glucose to whole cells results in the formation of labeled lipids(5). By contrast, radioactive glucose is very feebly incorporated into lipids in homogenates prepared from these cells. Furthermore, preparation of homogenates in distilled water, rather than in KCl-phosphate buffer, yields completely inactive extracts. Whole cells, however, are fully active in distilled water. Finally, inspection of the table shows that acetate is usually incorporated to a greater extent into the non-saponifiable fraction than into the fatty acids. This situation is exactly reversed when whole cells are used.

When the non-saponifiable fraction was treated with digitonin to precipitate the sterols, the isolated digitonides were found to contain radioactivity. This observation, obtained in 2 separate experiments, suggests the incorporation of acetate into ergosterol since the sterol fraction of this organism is composed almost exclusively of this compound(7).

Oxygen uptake was not a reliable index of the extent of acetate incorporation since some preparations with high Qo_2 values were less efficient in converting acetate to lipids than others with lower rates of oxygen uptake. The addition of succinate to the respiring homogenates stimulated oxygen consumption but had no enhancing effect on incorporation of acetate into non-saponifiable matter. Indeed, succinate was found to depress incorporation into this fraction, while it had no such effect on the formation of fatty acids.

Summary. Cell-free preparations, capable of incorporating acetate into fatty acids and non-saponifiable lipids, have been obtained from the yeast, *Saccharomyces cerevisiae.*

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Assay Method for the "Fibrin-Stabilizing Factor." (21711)

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Previous studies have established the fact that fibrin obtained by clotting pure fibrinogen with purified thrombin is soluble in 30% (w/v) urea, while the normal plasma clot does not dissolve in the same solvent(1,2,3). It has also been shown that the plasma clot is a mechanically stronger network than fibrin (4,5). Besides calcium ions, a thermolabile component of platelet-free plasma is necessary for the formation of a urea-insoluble clot. This component, called "fibrin-stabilizing factor" (FSF)* can be separated from fibrinogen, and is concentrated in a globulin fraction of plasma (mainly α-globulins) as prepared by the low temperature-ether fractionating technic(6,10).

The present studies were undertaken in order to establish a routine laboratory assay for measuring the FSF activity of plasma, and to determine its level in normal individuals.

Materials and Methods. Fibrinogen was purified from human or bovine plasma by refractionating the crude product obtained by the method of Ware *et al.*(8) with 0.25 saturated ammonium sulfate and redissolving the precipitate in 0.05% (w/v) ammonium carbonate. The fibrinogen was filtered through a Seitz-filter, lyophilized and stored in a deep freeze. When the fibrinogen was made into a solution, Michaelis' veronal buffer of pH 6.9 was added, to give a 1% (w/v) protein solution. We wish to thank Dr. C. Brambel for a supply of human fibrinogen (97% clottable). *Bovine or Human Thrombin* were obtained from prothrombin by activation in concentrated sodium citrate(9). The active enzyme was dialyzed against distilled water, and was diluted to contain about 2,000 units per ml. *Plasma* was separated from blood collected in Na-oxalate (9 vols. of blood to 1 vol. of 2% (w/v) oxalate), and was immediately placed in a deep freeze. It was tested for FSF activity within a week. The method described by Lorand(10) was used for *estimating the FSF activity* of plasma. To 1 ml of fibrinogen solution, various amounts of plasma and saline (0.9% w/v) were added to give a total volume of 3.9 ml. This was followed by the addition of 1 ml $CaCl_2$ (1% w/v) and immediate mixing of 0.1 ml thrombin. Clotting started within 15 seconds. Using bottles of about 5 cm inside diameter, the clots had a total volume of 5 ml and had the appearance of an opaque gel film. After waiting 15 minutes for the full development of the clot network, 5 ml urea (60% w/v) was added, and penetration of the gel was effected by shaking the bottles at 37°C, about 40 times per minute. After 24 hours the flasks were inspected for disappearance of the clots. The undissolved clot can be washed repeatedly with saline and water, dried and weighed, or simply classified by visual judgment, to range from zero, to +, 2+, 3+, or 4+, depending on its size. This latter subjective assessment seems to give remarkably uniform results from one worker to another. Under the above conditions a fibrin clot containing no FSF dissolved in urea within the first half hour. The amount of clot which remained undissolved was proportional to the amount of active FSF added. When testing plasma for FSF activity, a series of dilutions were investigated and the highest dilution which was still effective in producing a visible, urea-insoluble clot was taken as an estimate of the FSF activity of the particular plasma. This critical concentration of FSF activity is sharply defined (Table I). The same fact is borne out by the recent results of

* For historical background see ref. 6. The term plasma clot was adopted for the urea-insoluble type and fibrin for the urea-soluble clot. Terms previously used in the literature(7) such as Ca-fibrin (calcium fibrin) and T-fibrin (thrombin fibrin) are obviously not revealing, since primarily the presence of FSF and not that of Ca or thrombin alone decides the solubility properties of the clot. FSF has been referred to by others(5,11) as "Laki-Lorand or L-L factor" or "urea-insolubility factor".

TABLE I. Assay of FSF Activity of Plasma.

Plasma added to test, ml	Urea-insoluble clot remaining
.6	4+
.5	4+
.4	4+
.3	3+
.2	2+
.1	+
.05	0
—	0

Loewy and Edsall(11) using a similar method. For correct evaluation of the FSF activity test, there are 2 necessary controls. 1. A clot obtained in the absence of plasma must be urea-soluble, to show that the fibrinogen employed is free of FSF. 2. A urea-insoluble clot produced in the presence of relatively large concentrations of normal plasma (0.2 ml or more) must show that the fibrinogen used in the assay is capable of forming the urea-insoluble type of clot. Fibrinogen stored for long periods of time (more than 6 months) lost the ability to produce a urea-insoluble clot even though otherwise normal clotting occurred upon the addition of thrombin. Similar findings have been reported by other authors(11,12). Fibrinolysin(10) cannot act at the high urea concentration employed in the test. Thus by limiting the waiting period between the addition of thrombin and urea to 15 minutes, the unwanted interference by fibrinolysin has been greatly minimized.

Results. In a survey of 20 healthy individuals the following plasma concentrations were assayed in the urea-solubility test outlined above: 0.05; 0.1; 0.2; 0.3; 0.4 and 0.5 ml. Whenever 0.05 ml of plasma was employed, no urea-insoluble clot was left after 24 hours whereas small visible clots always were found if 0.1 ml or more plasma was assayed. Thus we concluded that under our experimental conditions, the critical concentration of FSF activity lies around 0.1 ml in normal individuals. This critical value of FSF in normal plasma is in good agreement with the approximately 0.2 ml found in samples of citrated pooled human plasma as tested in combination with a purified preparation of ether-fractionated fibrinogen(10).

An additional 110 people (hospitalized patients mainly with blood dyscrasias) were tested for critical FSF activities of their blood plasmas with the following results: 95 gave a value of 0.2 ml; 3 persons 0.3 ml; 1 person 0.4 ml; and in 11, the critical FSF concentration was manifest only if 0.6 ml or more plasma was employed in the test. Thus, individuals could be found with relative lack of FSF activity, but our studies are not sufficiently complete as yet, to draw a correlation between lowered FSF values and clinical disease. A major complication in evaluating the results seems to be the finding that plasmas previously shown to be deficient in FSF, give near normal values after storage in the deep freeze for 2-3 months.

Having developed an adequate method for assaying FSF activity in plasma, and having established that the critical normal value lies below 0.2 ml, we could follow changes of apparent FSF concentration in the course of the blood coagulation process. Blood was collected without an anticoagulant, immediately centrifuged, and the plasma pipetted off before clotting took place. Samples were tested for FSF activity immediately, and also at different intervals after clotting took place. The results are summarized in Table II.

From the data in Table II it is evident that there is a dramatic drop of FSF in serum as soon as clotting takes place. This finding brought up the possibility that the FSF was removed from the serum by adsorption to the clot network, as indeed was suggested earlier (4). The following experiment showed that FSF has a high affinity for fibrin. Two identical samples of oxalated plasma were clotted by the addition of thrombin. The resulting clots were washed with large volumes of physiological saline at 0°C for 24 hr. Then

TABLE II. Changes of FSF Activity during Coagulation of Blood.

	Time after drawing blood,* min.	Critical FSF value, ml
Plasma	2	.1
Serum	14	1.0
	36	2.0
	84	>2.9

* Clotting took place between 4 and 10 min.

one clot was kept in saline for another day, while the second clot was transferred into a solution of 1% (w/v) $CaCl_2$. Both clots were placed afterwards in 30% (w/v) urea, and it was found that the first clot dissolved entirely in this solvent, whereas the second, (incubated with calcium) became insoluble. This observation indicated that FSF remained attached to the fibrin even after exhaustive washing. Furthermore, it showed that urea-soluble clots could be converted into the insoluble type by incubation with calcium if FSF was present. The latter finding was also reported by Loewy and Edsall(11).

Discussion. Besides the academic interest in recognizing a new functional principle in plasma, studies on the "fibrin-stabilizing factor" may have some profound significance with regard to the practical problems of blood coagulation. It has already been shown that the plasma clot is a stronger network than fibrin(4,5). Also there are indications that plasma clots are more slowly attacked by fibrinolysin than fibrin clots(13).

The formation of a stronger, more resistant network than fibrin during normal blood coagulation no doubt prejudices the mode of the retraction of the clot. It seems reasonable to suppose that, to a certain extent, retraction can take place better in a gel system in which the particles are well cross-linked and oriented. Thus, the existence of a closely knit gel would seem to be a prerequisite for adequate clot retraction. This assumption would suggest that the plasma component, known to participate in clot retraction in conjunction with platelets(14,15), may turn out to be identical with the "fibrin-stabilizing factor." It has been reported earlier(6,10) that about 75% of the total "fibrin-stabilizing factor" activity of plasma can be recovered in a globulin fraction (mainly α-globulins) using the low temperature-ether fractionating method. The work of Savitsky suggests that the clot retracting plasma component(15) may also be concentrated in similar globulin fractions. It remains to be seen, however, whether the clot retracting component is identical with the "fibrin-stabilizing factor." Such an identity, though suggestive, could be established only after full characterization of either substance, but in any case the possibility of relationship is worth attention.

The present investigation revealed a method to assay the FSF activity of plasma and it was found that normal individuals show a critical value of about 0.2 ml plasma concentration for producing a urea-insoluble clot in the standard test. Critical values greater than 0.2 ml were taken to indicate a FSF deficiency.

The FSF activity of plasma was shown to be greatly diminished after clotting is allowed to take place, because FSF has a strong affinity to be adsorbed to fibrin. Moreover, clots which are soluble in urea can be converted into the insoluble type by incubating with $CaCl_2$ in the presence of FSF. The strong affinity of FSF to fibrin might indicate that the factor is a cementing partner of fibrin in forming the clot, but the possibility of it acting as a catalytic agent in producing stronger cross linkages, cannot be ruled out. Recent results by Loewy and Edsall(11) point to the latter possibility in that they were able to enhance the production of urea-insoluble clots by certain SH compounds. One has to bear in mind, however, that artificially produced insoluble clot networks may not be identical with the natural plasma clot gel.

Summary. 1. A test is described to measure the FSF activity of plasma. 2. Under the conditions defined, normal human plasma gives a critical FSF value in amounts between 0.1 and 0.2 ml. 3. There is an indication for elevated FSF values under pathologic conditions. 4. The FSF activity of serum is greatly reduced as compared with levels in plasma, because FSF is strongly adsorbed to fibrin. 5. It is suggested that the physiological formation of a urea-insoluble plasma clot is a prerequisite for normal clot retraction, and that the plasma component needed for syneresis may turn out to be identical with FSF.

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Phospholipide Metabolism in Various Tissues of Cholesterol-Fed Rabbits.* (21712)

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To investigate the possible relationship between phospholipide turnover rate and tissue-lipide accumulation, we have here studied the effect of cholesterol feeding on liver, lung, small intestine, kidney, muscle, aorta and plasma.

Methods. Albino rabbits were maintained on 100 g Purina rabbit chow per day; cholesterol-fed rabbits received daily 100 g of this diet containing 1 g cholesterol‡ dissolved in 2.8 g fat (Humko).§ After 5 months on these diets normal and experimental animals were injected intravenously with 0.5-1.0 mc of P^{32}. Six hours after the administration of the radioactive dose the animals were bled, killed with nembutal and samples of liver, lung, kidney, small intestine, thoracic aorta and muscle were taken for analysis. The tissues were weighed, ground and extracted with ethanol, ethyl ether and petroleum ether in a manner previously described(1). Phospholipide P^{31} and P^{32} were determned on aliquots of the final extracts. In some experiments the defatted liver residues were dried over calcium chloride to constant weight.

Results. In Table I are presented the lipide phosphorus concentrations and specific activities in the control and cholesterol-fed animals. The results have been divided into 2 groups. The first series, presented at the top on Table I, comprises a study on 5 control and 5 cholesterol-fed animals. The results obtained in this group failed to show any decisive changes in phospholipide concentrations or specific activities of the 5 tissues examined. However, an inspection of the data revealed that the standard errors of the means of the specific activities were so large that the experiments did not furnish definite evidence on the effect of cholesterol feeding on the phospholipide turnover of these tissues. To enlarge the series of animals the experiments were repeated with certain slight modifications. The rabbits in the second group, presented in the bottom part of Table I, were matched according to weight at the beginning of the feeding period. In addition the total organ weights were recorded. In order to facilitate the grinding of lung and aorta a small amount of sand was added to these tissues. To eliminate the possibility that the sand introduced phosphorus or a phosphorus-like contaminant,

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‡ Cholesterol was kindly donated by Merck and Co.

§ Rabbits in series 1 received Humko vegetable fat. As a result of a change in product those in series 2 received an unknown and variable mixture of animal and vegetable fats, sold under the same name.

TABLE I. Concentrations and Specific Activities of Phospholipide in Various Tissues of Cholesterol-Fed and Normal Rabbits 6 Hr after P^{32}.*

	Phospholipide P, mg/g tissue		No. of animals		Phospholipide specific activity†		Organ wt, g	
	Control	Cholesterol fed	Control	Cholesterol fed	Control	Cholesterol fed	Control	Cholesterol fed
				Series 1				
Lung	.56 ± .12	.73 ± .006	5	5	17.7 ± 2.1	11.1 ± .5		
Small int.	.65 ± .02	.68 ± .05	5	5	18.9 ± 1.7	15.9 ± 1.4		
Kidney	.96 ± .01	1.03 ± .02	5	5	29.8 ± 3.1	23.9 ± 1.7		
Muscle	.29 ± .03	.27 ± .02	5	5	.51± .08	.80± .18		
Liver	1.11 ± .12	1.13 ± .07	5	5	24.8 ± 5.3	21.0 ± 4.6		
				Series 2				
Lung	.94 ± .04	1.07 ± .05	5	5	8.4 ± .8	8.5 ± .8	13.1	12.6
Small int.	.65 ± .05	.69 ± .09	5	5	24.9 ± 4.1	20.8 ± 2.6	56	62
Kidney	1.01 ± .05	1.08 ± .04	5	5	21.1 ± .9	18.4 ± 1.4	15.3	17.7
Muscle	.27 ± .004	.25 ± .007	5	5	.37± .05	.44± .06	—	—
Liver	1.30 ± .04	1.40 ± .05	7	7	12.9 ± 1.2	12.4 ± 1.4	74	134
Aorta	.25 ± .02	.66 ± .08	7	7	7.2 ± 1.0	5.7 ± 1.1	.59	1.69
Plasma	.026± .004	.20 ± .02	7	7	8.7 ± 1.9	3.2 ± .6	—	—

* Means ± stand. errors (stand. dev./$\sqrt{N}$). Stand. dev. was determined from the range(2).
† Specific activity = % of injected P^{32}/g of phospholipide P.

the sand was taken through the procedure and tested phosphorus free. Duplicate analyses showed that the use of sand with liver and lung did not increase the apparent phospholipide content of these tissues. The higher phospholipide content of the lungs in the second group must, therefore, reflect a real difference between the 2 groups. The apparent difference between the liver phospholipide specific activities of the 2 control groups was not found to be significant at the 5% level of confidence. Except for the fact that the second set of experiments provided more precise results, the effect or lack of effect of cholesterol in the 2 sets of experiments was the same. An analysis for homogeneity of variance indicated, however, that the data of the 2 sets of experiments should not be combined.

Cholesterol feeding produced 3 types of changes: 1) in phospholipide concentration 2) in phospholipide specific activity (a measure of the fraction of the total organ's phospholipide turned over) and 3) in organ weights. The product of these 3 factors gives an estimate of the rate of phospholipide synthesis in the whole organ. Lung, small intestine, kidney and muscle do not appear to show any effect of cholesterol feeding on the phospholipide turnover rate. Similarly, in the liver there does not appear to be any change in the concentration or specific activity of the phospholipides; however, the liver weight of the cholesterol-fed animals was nearly twice as great as that of the controls. This increase in liver weight is not the result of fatty infiltration of this organ since the dried defatted residues amounted to 21.0% and 19.1% of the wet weight of the liver of the control and cholesterol-fed groups, respectively. Since the body weights of the 2 groups were practically the same, the feeding of cholesterol appears to result in a marked increase in relative liver mass. In the absence of any effect of cholesterol feeding on the specific activities of liver acid soluble phosphates(1) one may thus conclude that the rate of phospholipide synthesis per unit of liver protoplasm was not increased by cholesterol feeding but that in view of the increased tissue mass the synthesis per whole liver was nearly doubled.

Aorta and plasma, in agreement with our previous observations(1), show marked increases in phospholipide turnover rates by virtue of their increased phospholipide concentrations (and increased organ weight in the case of aorta), even though the specific activities in the cholesterol-fed animals were lower. In the whole thoracic aorta in contrast to liver,

it appears that the increased phospholipide is partly the result of an increase in anabolic activity of the aortic protoplasm. Per whole thoracic aorta there is about a 7½-fold increase in total phospholipide, a 6-fold increase in the amount of radioactive phospholipide (index of synthesis) but only a 3-fold increase in fresh tissue weight. However, until more is known about the site of increased phospholipide synthesis in the atheromatous aorta, the mechanism whereby the amount of radioactive phospholipide in this aorta is increased must remain the object of speculation.

Discussion. Our previous finding(1) that phospholipide synthesis in the atheromatous aorta of rabbits is markedly increased raised the question whether the feeding of cholesterol promotes the synthesis of tissue phospholipides in general or whether the aorta exhibits a specific response to cholesterol feeding. There appears to be no effect of prolonged cholesterol feeding on the phospholipide turnover of lung, small intestine, kidney, or muscle, tissues which do not exhibit any gross changes in lipide content. On the other hand, aorta, plasma, and liver, 3 "tissues" where large lipide accumulations take place, also exhibit marked to moderate increases in phospholipide turnover. One might, therefore, speculate that some relation exists between the accumulation of lipides in a tissue and the stimulation of phospholipide turnover. In view of other work on the possible relation of phospholipide turnover and fat oxidation(3) one might be tempted to seek for an explanation of the observed parallelism in lipide accumulation and phospholipide turnover along these lines. The tissue may respond to the excess lipide by increasing its rate of lipide oxidation, a process in which phospholipides appear to participate. Alternatively, one might think of phospholipides as emulsifying agents and colloid stabilizers. The frequently expressed idea that plasma phospholipides serve to maintain the plasma cholesterol in solution may have a parallel in other tissues. When, for example, excessive amounts of cholesterol appear in the aorta of the rabbit the tissue may increase its phospholipide synthesis in an "attempt" to solubilize the extra cholesterol.

In contrast to our finding of a higher phospholipide turnover in the whole liver of the cholesterol-fed rabbit, Perlman *et al.*(4) and more recently Clément *et al.*(5) found that the feeding of cholesterol to rats depressed the synthesis of liver phospholipides. The latter group of workers even showed that the depressed phospholipide turnover persisted as long as one month after the cholesterol had been removed from the diet. The apparent disagreement between the response of the rat and the rabbit to cholesterol feeding may constitute a species difference. The rabbit, susceptible to cholesterol atherogenesis, increases its rate of liver phosphatide synthesis whereas the rat, known to be resistant to this disease, responds to cholesterol with a decrease in phospholipogenesis. Pilgeram and Greenberg (6) on the other hand conclude from their studies on the incorporation of C^{14}-labeled ethanolamine into phospholipides of rat liver slices that the rate of lecithin synthesis is increased by cholesterol. A study of the effect of cholesterol on the intermediate products of phospholipide synthesis may throw some light on the apparent disagreement between the C^{14} and the P^{32} data. The further study of the metabolism of phospholipides in different species may furnish an important link in our understanding of atherogenesis.

Summary. Rabbits maintained for 5 months on an atherogenic diet showed a marked increase in the incorporation of radioactive phosphate into the phospholipides of aorta, plasma, and the whole liver. In contrast the feeding of cholesterol and fat did not appear to have an appreciable effect on phospholipide synthesis in lung, small intestine, kidney, and muscle. The livers of the cholesterol-fed animals showed a marked increase in tissue mass over and above that caused by fatty infiltration; other organs except aorta did not exhibit significant changes in weight. A possible relationship between fatty infiltration and phospholipide turnover is discussed.

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I^{131} Blood Levels Correlated with Gastric Emptying Determined Radiographically. I. Protein Test Meal. (21713)

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(Introduced by Barnes Woodhall.)

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Iodine 131 blood levels were measured in normal subjects following ingestion of a radioactive labeled protein test meal. The distribution of the meal in the gastrointestinal tract, as determined radiographically, was correlated with the blood levels. This study was undertaken as the first of a series of investigations using radioisotope technic in the evaluation of gastrointestinal function both in health and disease.

Material and methods. A. Human subjects. Thirty-six subjects were studied, 24 males and 12 females, either Duke University students or hospital employees, all in good health. Each was given 20 drops of Lugol's solution 3 times daily for 3 days before beginning the test. The subject, having fasted for 6 hours, ingested a test meal as follows: 50 μc (0.1 cc) of I^{131} labeled human serum albumin,† ½ g Knox dietary gelatin per kilo body weight, and 225 cc of tepid water. In 17 of the studies 20 g of barium sulfate were added to the test meal. This was prepared immediately before ingestion by mixing the ingredients in a 400 cc beaker. Following ingestion of the test meal, the beaker was rinsed twice with 50 cc of water and the subject drank the rinse, but otherwise fasted throughout the test. The beaker was then assayed for residual radioisotope content, and this was taken into consideration in the final analysis. All subjects, except 2, were ambulatory or seated during the test. Two cc of venous blood were withdrawn from 30 subjects at 10, 20, 30, 45, 60, 90, 120, and 180 minutes after ingestion of the test meal. In 6 subjects the blood samples were taken at intervals of 4 minutes throughout the 3-hour period. These samples were obtained by a single vena puncture, using a continuous infusion of normal saline. Not more than 200 cc of saline were administered to any one subject during the test, and care was taken to obtain an undiluted blood sample. Each sample was placed in a separate tube and assayed for radioactive I^{131} content in a calibrated, sodium iodide, thallium activated, scintillation crystal well counter. The total blood volume was assumed to be 7.2% of body weight. Using the measured radioactivity of the 2 cc sample and the calculated total blood volume, the total radioactive I^{131} blood level was determined and expressed as a per cent of the ingested material. From these data a curve was constructed giving the relationship of instantaneous radioactive I^{131} blood levels to time after ingestion of the test meal. The progress of the test meals containing barium sulfate was followed by roentgenograms taken at 15, 30, 60, 90, 120 and 180 minutes after ingestion.

B. Dogs. Fifteen healthy dogs were stud-

* Trainee of National Institute of Health, Division of Metabolism and Arthritis.

† This material was obtained from Abbott Laboratories under authorization of the Atomic Energy Commission.

ied in a similar fashion with the following exceptions: 1) Potassium iodide tablets were given instead of Lugol's solution; 2) barium was omitted and no x-rays were taken; 3) the test meal was administered by stomach tube, and 4) the samples of venous blood were taken at 15, 30, 45, 60, 90, 120 and 180 minutes after administration of the meal. Five dogs were studied in the following manner: Under local anesthesia, a nylon catheter was passed around the pylorus. The ends of the catheter were brought outside the abdominal cavity and tied over a glass rod. The pylorus, thus, could be occluded by tightening the catheter. These dogs were studied with and without the pylorus occluded.

Results. Thirty-one of the 36 subjects were accepted as normal controls. For reasons which will be discussed later, 5 of the subjects were considered abnormal during the test period and the results in these cases will be treated separately. The average blood level and standard deviation for each point of observation are shown in Fig. 1. Fig. 2 shows a typical curve from one of the 6 subjects from whom blood samples were taken at 4-minute intervals. It will be seen that during short intervals there are no wide variations in the blood levels and a smooth, continuous curve results.

Ten of the 15 dogs were used as normal controls and the test was repeated in each animal after an interval of one to 2 weeks.

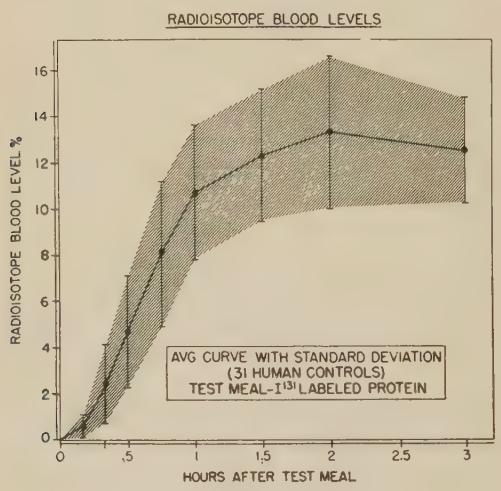

FIG. 1.

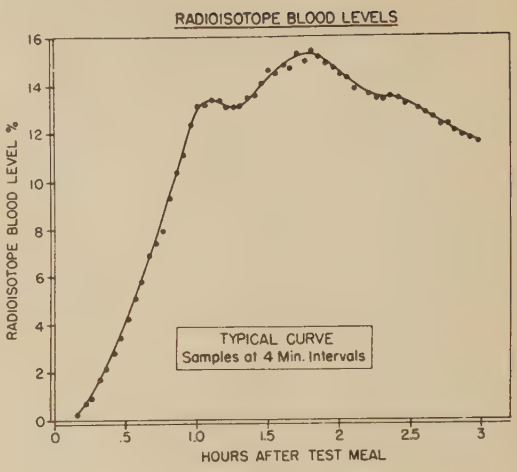

FIG. 2.

The average radioisotope blood level and standard deviation for each point are shown in Fig. 3. There is a close similarity in the shape and height of the resultant curves in the human and dog studies.

Seventeen subjects whose test meal contained barium sulfate were studied radiographically to determine the effect of gastric emptying on the radioisotope blood levels. Delayed gastric emptying resulted in a delay in the time at which the blood levels began to rise. Graphically this caused a shift to the right in the resultant curve. The addition of barium sulfate to the test meal produced no significant change in the blood levels.

The results of the study in dogs with the pylorus occluded confirmed the findings of the above mentioned radiographic study. These surgically prepared dogs had radioisotope blood levels within the normal range when the pylorus was patent. However, when the pylorus was occluded by tightening the catheter, there was little or no absorption.

As indicated above, 5 of the 36 subjects were eliminated from the normal control group prior to determination of the blood levels. Four fainted and one subject was changed from the supine to the upright position during the test. The radioisotope blood levels dropped during the period of syncope and rose in the one subject whose position was changed.

Discussion. Previous studies would indicate that the blood levels of radioactive material

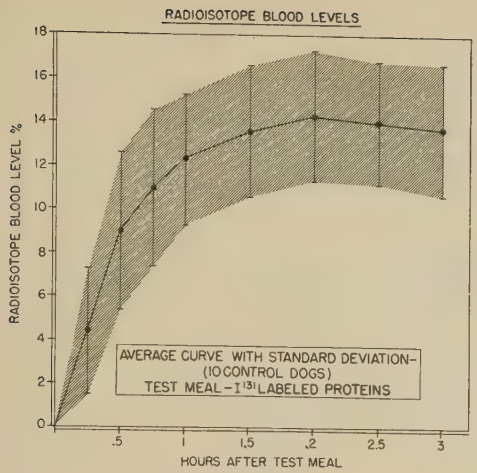

FIG. 3.

following ingestion of a test meal containing radioactive I^{131} tagged albumin, differ significantly in patients having pancreatic disease as compared with those of normal individuals(1). On the contrary, the blood levels following ingestion of an I^{131} test meal, in children having cystic fibrosis of the pancreas, were so variable that no conclusions could be drawn. However, a significant increase in fecal excretion of the radioisotope was observed and was thought to be a more reliable test in the diagnosis of this disease(2,3).

The present study shows clearly that following ingestion of an I^{131}-labeled protein test meal by normal subjects, the radioisotope blood levels, when depicted graphically, give a characteristic and reproducible curve. In the course of this investigation the need for definite knowledge of the relationship of gastric emptying to the radioisotope blood levels became apparent. A delay in gastric emptying, as determined radiographically, is accompanied by a delay in the time at which the blood level begins to rise. Since the time of initial rise in the blood level is directly dependent upon gastric emptying, radiographic examination is essential in studies of this nature. Further evidence of the importance of gastric emptying is seen in the results obtained in dogs studied with and without pyloric occlusion.

The radioisotope blood levels in the 4 subjects who fainted were depressed during the period of syncope. In 3 of these subjects gastric emptying had taken place normally prior to the onset of fainting and the test meal could be demonstrated throughout the upper intestinal tract. However, in the fourth subject, fainting took place at the beginning of the test before blood samples could be obtained. An x-ray film taken 30 minutes later showed poor emptying and the radioisotope blood level at 40 minutes after beginning the test was significantly lower than normal. Poor emptying of the stomach was demonstrated radiographically in one subject while supine. When the subject was changed to the upright posture, there was more rapid emptying and a concomitant rise in blood levels was observed.

It should be emphasized that even in normal subjects certain factors may significantly alter the radioisotope blood levels. In this study delayed gastric emptying, fainting, and position were found to be important. Failure to take into consideration the factors mentioned above could lead to erroneous interpretation of the results.

Conclusions. 1. A characteristic and reproducible curve of radioisotope blood levels was obtained in normal humans and normal dogs following an I^{131} labeled protein test meal. 2. Radiographic determination of gastric emptying is necessary for the proper interpretation of data. 3. Delayed gastric emptying significantly altered the radioisotope blood levels. 4. Fainting and change in position may alter radioisotope blood levels.

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I^{131} Blood Levels Correlated with Gastric Emptying Determined Radiographically. II. Fat Test Meal. (21714)

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This report concerns the use of an Iodine 131 labeled fat test meal in the study of digestion and absorption in normal subjects. Gastric emptying was determined radiographically and correlated with the radioisotope blood levels. Problems in fat digestion and/or absorption have been under investigation at Duke Hospital for many years. Hanes(1) reported extensively on the sprue syndrome, emphasizing important laboratory and clinical features. Ruffin *et al.*(2) utilized vit. A and carotene absorption tests in the evaluation of intestinal function and more recently demonstrated the efficacy of the wheat free diet in treatment of sprue(3). Fox *et al.*(4) conducted fat balance studies in normal and abnormal patients. Hendrix, Black-Schaffer, and Handler(5) described the pathological and clinical features of Whipple's disease. Hornsby and Baylin(6) recently pointed out certain roentgenological differences in the small intestinal pattern in patients with pancreatic steatorrhea and sprue.

This study of the normal was undertaken in preparation for the further investigation of certain physiological aspects of fat digestion and/or absorption in normal and abnormal subjects.

Material and methods. A. Human subjects. Thirty-three Duke University students were used as normal controls, 31 of whom were males. The age range was from 18 to 25 years. Preparation for the test was the same as in the previous study, in which I^{131}-labeled protein was used(7). The test meal consisted of the following: 50 μc (0.1 cc) of I^{131}-labeled glycerol trioleate,† ½ cc of peanut oil per kilo of body weight emulsified in an equal amount of water, and 2-3 cc of Tween 80. In 25 subjects, 20 g of barium sulfate were added to the test meal, and the ingredients of the meal were mixed in a Waring Blender. Following ingestion of the meal, the beaker was rinsed twice with 50 cc of water, and the subject drank the rinse and fasted during the test period. The beaker was then assayed for residual radioisotope content, and this was taken into consideration in the final analysis. All subjects were ambulatory. Two cc of venous blood were withdrawn from 8 subjects at 60, 90, 120, 180, and 240 minutes after ingestion of the meal. In the remaining 25 subjects, blood samples were taken at 60 minute intervals for a 6-hour period. Each sample was placed in a separate tube and assayed for radioactive I^{131} content in a calibrated, sodium iodide, thallium activated scintillation crystal well counter. The total blood volume was assumed to be 7.2% of body weight. Using the measured radioactivity of the 2 cc sample and the calculated total blood volume, the total radioactive I^{131} blood level was determined and

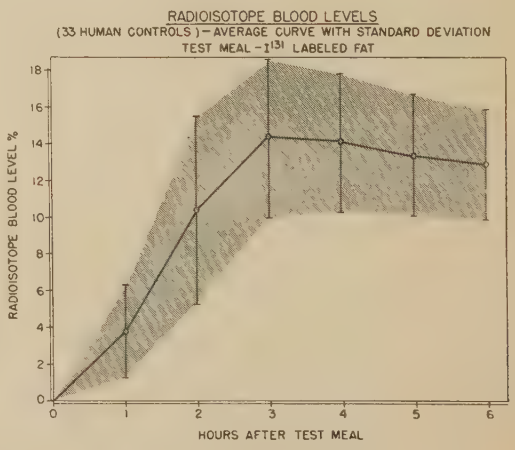

FIG. 1.

* Trainee of National Institute of Health, Division of Metabolism and Arthritis.

† This material was obtained from Abbott Laboratories under authorization of the Atomic Energy Commission.

expressed as a percentage of the ingested material. From these data a curve was constructed showing the relationship of instantaneous radioactive I^{131} blood levels as a function of time. The progress of the test meal in 4 subjects was followed by roentgenograms taken at 60, 90, 120, 180, and 240 minutes after ingestion. Twenty-one of the remaining subjects had roentgenograms taken at intervals of either one or 2 hours during a 6-hour period. In 24 subjects the urine was collected for a period of 6 hours following ingestion of the meal and assayed for its radioisotope activity. In 6 subjects all feces were collected for 48 hours and analyzed for I^{131} content.

B. Dogs. Eleven healthy dogs were studied in a similar fashion with the following exceptions: (1) Potassium iodide tablets were given instead of Lugol's solution; (2) barium was omitted and no x-rays were taken; (3) the test meal was administered by stomach tube, after which the tube was immediately withdrawn; (4) the samples of venous blood were taken at 30, 60, 90, 120, 150, 180, 240, 300, and 360 minutes after administration of the meal; and (5) analysis of the urine and feces was omitted.

Results. The average radioisotope blood level and standard deviation for each point of observation in 33 normal patients are shown in Fig. 1. The radioisotope content of urine excreted during the 6 hours following ingestion was determined and showed considerable variation. Analysis of the radioisotope content of the feces in 6 subjects showed less than 2% of ingested material in every case. No barium was observed by x-ray in any subject after 48 hours, presumably indicating that the test meal had been eliminated from the intestinal tract.

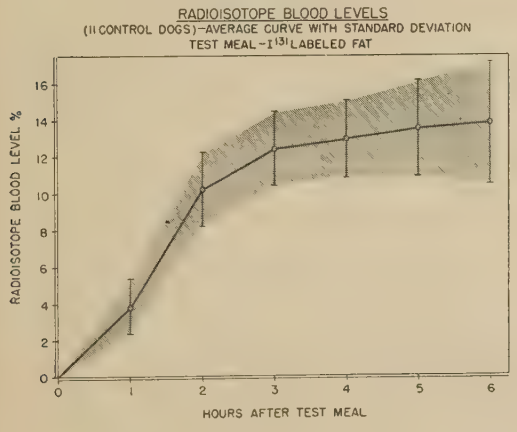

FIG. 2.

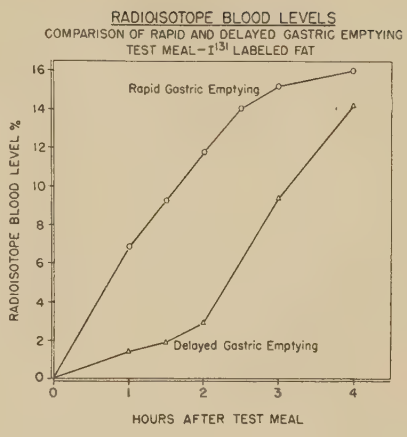

FIG. 3.

Eleven dogs were used as normal controls. The average radioisotope blood level and standard deviation for each point are shown in Fig. 2. There is a close similarity of the results in man and in dogs.

In the 25 subjects who ingested a test meal containing barium, x-ray studies were performed in order to correlate gastric emptying and radioisotope blood levels. The time of rise of the radioisotope blood level was found to be dependent upon the time of gastric emptying. In Fig. 3 a typical curve of the blood levels associated with rapid gastric emptying is compared with one associated with delayed emptying. As might be expected, gastric emptying of the fat test meal was slower than that of the protein meal used in the previous study(7). The resultant curves in the protein and fat study (Fig. 4) show the effects of this difference.

Discussion. A characteristic and reproducible curve of radioisotope blood levels was obtained in normal subjects after ingestion of a labeled fat test meal. The shape and height of this curve was found to be dependent upon the quantity of peanut oil given per kilo of body weight, and variations from the amount used (½ cc per kilo of body weight) altered the resultant curve. There-

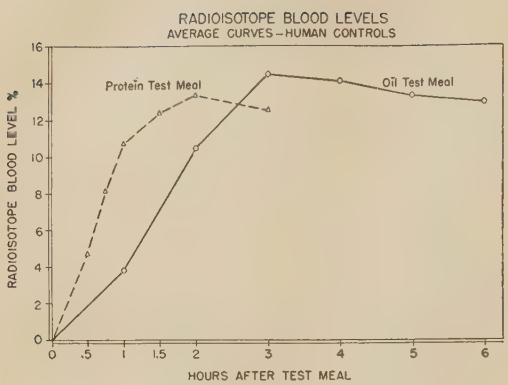

FIG. 4.

fore, the amount of oil given must be considered critical.

The determination of the time of gastric emptying is necessary for the proper evaluation of the results if the technic is to be applied clinically. In some instances the resultant curves of radioisotope blood levels in normal subjects are low for several hours and then reach normal values. Thus far our studies have shown that this is associated with slow gastric emptying. If in the clinical application a flat curve is obtained, this could reflect either poor intestinal function or faulty gastric emptying. Since in certain diseases gastric retention for a period of more than 6 hours does occur, the radiographic study is necessary to rule out this possibility; otherwise, false conclusions may be drawn.

Shingleton *et al.*(8) have applied this technic in the study of a group of patients with pancreatic disease and demonstrated that in all instances the radioisotope blood levels were significantly lower than normal. Application of this technic to patients with other gastrointestinal diseases is in progress.

It is of interest that Borgstrom(9) in his work with rats obtained similar radioisotope blood level curves, although using different materials and technics. Thus, the similarity of results in rats and dogs and in humans lends validity to a further investigation of pertinent gastrointestinal physiological processes in animals as a guide to gastrointestinal function in humans.

Although the studies of the urine did not suggest a potential clinical application, the consistently low fecal excretion of I^{131} indicates that this may prove of value as a clinical test.

Conclusions. 1. A characteristic and reproducible curve of radioisotope blood levels was obtained in humans and dogs following an I^{131} labeled fat test meal. 2. Radiographic determination of gastric emptying is necessary for the proper interpretation of data.

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Influence of Rauwiloid®, Alkaloidal Extract of *Rauwolfia serpentina*, on Veratrum-Induced Emesis in Dogs.* (21715)

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Rauwiloid has been demonstrated by various indices(1-3) to exert a sedative effect in laboratory animals at experimental dosage and a tranquilizing action in man at clinical dosage. It is conceivable that this may also include depression of receptor areas in the central nervous system concerned with veratrum emesis. Such a concept is supported by the recent clinical reports of Finnerty(4), La Barbera(5), and Wilkins(11) citing evidence that the incidence of gastrointestinal side-effects in hypertensive patients receiving veratrum medication was markedly reduced when Rauwiloid was added to the therapeutic regimen. Previous attempts to control the nausea and vomiting due to veratrum have not been encouraging. Experimentally, Swiss(6) has demonstrated that premedication of unanesthetized dogs with compounds such as atropine, scopolamine, tetraethylammonium chloride (TEA), ephedrine, dimenhydrinate (Dramamine) and methantheline bromide (Banthine) failed to diminish the emetic response to veratrum—excepting where the dose of the premedicants themselves was high high enough to cause still other undesirable manifestations. Brand and his co-workers(7) have reported that chlorpromazine, while significantly elevating the emetic threshold to apomorphine in dogs, was ineffective against veratrum-induced emesis. The results of clinical attempts towards this end using combination veratrum-antispasmodic or barbiturate therapy have been equivocal.

It was the purpose of our experiments to observe the effect of premedication with Rauwiloid on the emetic response to veratrum in dogs.

Method. The ED_{50} of emesis for intravenously administered Veriloid®, a mixture of veratrum alkaloids, was determined on 45 dogs.† The drug was dissolved in 0.1% acetic acid solution and the final concentration adjusted so that the injected volume was 5 ml per dog. The injections were made rapidly intravenously and criterion of emesis was expulsion of stomach contents within one hour following drug administration. A total of 54 dogs (including controls) received Rauwiloid orally for 5 days at a dose of 0.5 mg/kg/day which dose has been demonstrated to produce sedation‡ in approximately 50% of animals (1). On the sixth day, these dogs were classified as either sedated or non-sedated, and an ED_{50} for Veriloid determined for each category. For comparison, the effect of sedative doses of phenobarbital sodium on emetic threshold to Veriloid was determined in 24 dogs. Twenty-five mg/kg phenobarbital sodium were administered intravenously and the ED_{50} for Veriloid determined one hour later. This dose of phenobarbital produced an apparently similar type of central nervous system depression in animals as did Rauwiloid; frank ataxia was not observed with either drug. In determining the ED_{50} for Veriloid, the dose range 17.1 to 28 μg/kg was employed. The 45 control animals were divided into 3 groups of 15 dogs each and tested at dose levels of 17.1, 21.3 and 23.8 μg/kg. Following 5-day medication with Rauwiloid, 26 of 54 animals were classified as non-sedated, divided into 3 groups of 9, 9, and 8 dogs each, and tested at doses of 21.3, 23.8, and 28 μg/kg. The remaining 28 sedated animals were also divided into 3 groups of 10, 8, and 10 dogs each and tested at the same dosages as the non-sedated dogs. The 24 animals premedicated with phenobarbital sodium

* A preliminary report was given at interim meeting of Soc. Pharmacol. and Exp. Therap., Univ. of Virginia, Sept. 1954.

† ED_{50} was calculated according to the method of Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. and Exp. Therap.*, 1949, v96, 99.

‡ The animals lacked their usual spontaneous motor activity but were readily aroused and capable of locomotion.

TABLE I. Effect of Rauwiloid or Phenobarbital Sodium Premedication on Emesis Induced by Veriloid in Dogs.

Dose Veriloid, μg/kg I.V.	Incidence of emesis: Control	After Rauwiloid p.o., 0.5 mg/kg/day × 5: Non-sedated	After Rauwiloid p.o., 0.5 mg/kg/day × 5: Sedated	After phenobarbital Na, 25 mg/kg I.V.
17.1	3/15	—	—	3/8
21.3	9/15	4/9	2/10	6/8
23.8	12/15	7/9	3/8	6/8
28.0	—	7/8	6/10	—
ED_{50} (μg/kg), 95% confidence limits	20.2 (18.7-21.8)	21.0 (18.4-23.9)	26.2 (22.8-30.0)	18.9 (16.3-21.9)
	Sedated / Control	Sedated / Non-sedated	Non-sedated / Control	Phenobarbital Na / Control
Slope ratio	1.08*	1.07*	1.02*	1.09*
Potency ratio (95% confidence limits)	130 (112-148) ($p < 0.01$)	125 (103-151) ($0.02 > p > 0.01$)	105 (90-133)	94 (80-111)

* No significant deviation from parallelism.

were divided into 3 groups of 8 dogs each and tested at the same dose levels as the controls.

Results. The ED_{50} (95% confidence limits) of Veriloid for the control group was 20.2 (18.7-21.8) μg/kg. Following 5-day premedication with Rauwiloid the ED_{50} for the non-sedated animals was 21 (18.4-23.9) μg/kg and the ED_{50} for the sedated animals was 26.2 (22.8-30) μg/kg (Table I). In dogs premedicated with phenobarbital sodium, the ED_{50} for Veriloid was 18.9 (16.3-21.9) μg/kg. Examination of the 4 dose-response lines revealed no significant deviation from parallelism (Table I, Fig. 1). The emetic threshold for Veriloid in the dogs sedated by premedication with Rauwiloid was elevated 30% over the control ($p < 0.01$) (Table I). Similarly, the emetic threshold in the sedated animals was 25% greater than in the non-sedated animals ($0.02 > p > 0.01$). On the other hand, there was no significant difference between the ED_{50} values of the control, non-sedated dogs premedicated with Rauwiloid or phenobarbital-premedicated dogs.

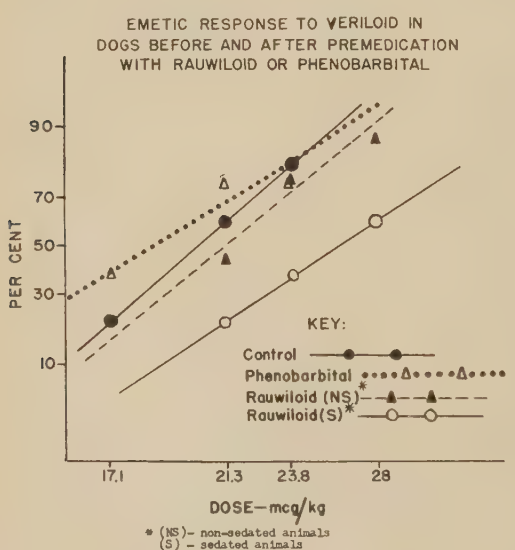

FIG. 1.

Discussion. The results indicate that Rauwiloid, in doses sufficient to produce discernible sedation, elevates the emetic threshold to Veriloid in dogs, whereas non-sedative doses are without this effect. Clinical interpretation of this observation will be affected by two considerations: First, the dose of Rauwiloid tested, 0.5 mg/kg/day, compares favorably, on a per kilogram basis, with the highest dose employed clinically, and is 8 times the recommended clinical dose. Secondly, the most frequent gastrointestinal complaint following veratrum is nausea, not vomiting, and the two are not necessarily correlated. Further, the production of emesis by rapid intravenous administration of Veriloid is not necessarily analogous to production of nausea by oral medication.

Elucidation of the mechanism of anti-emetic

activity, however, remains difficult because the site of emetic action for veratrum alkaloids in dogs has not been strictly defined. Christiansen and McLean(8), Marsh(9), and Swiss(6) have indicated that a central effect is possible. The experiments of Borison and Fairbanks (10) describe the nodose ganglion as essential for the emetic response to veratrum in cats. The ability of sedative doses of Rauwiloid to elevate the emetic threshold for Veriloid as opposed to the ineffectiveness of sedative doses of phenobarbital in this respect, argues against the premise that the anti-emetic activity of Rauwiloid may be due to a barbiturate-type depression of the central nervous system. In addition, the previously mentioned results of Swiss(6) and Brand(7) which demonstrated that pharmacologic agents of the following categories—parasympatholytic (atropine, scopolamine, Banthine), ganglionic-blocking (TEA), sympathomimetic (ephedrine), antihistaminic (Dramamine) and chlorpromazine failed to protect dogs against the emetic effect of veratrum, suggest the possibility that some other mechanism may be involved. Whether this is based on a selective depression of certain higher centers or on some other basis requires further investigation.

Summary. 1. Rauwiloid, a selected alkaloidal extract of *Rauwolfia serpentina,* administered orally for 5 days to dogs at a dose of 0.5 mg/kg/day, significantly elevated the emetic threshold to intravenously administered Veriloid in those animals which were sedated. No alteration in the emetic threshold was observed in non-sedated animals. 2. Premedication of dogs with intravenously administered phenobarbital sodium at a dose of 25 mg/kg which produced a degree of sedation apparently similar to that of Rauwiloid, was without effect on emesis induced by Veriloid.

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Suggestive Evidence of a Primary "Drinking Center" in Hypothalamus of the Rat. (21716)

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Although the existence of a hypothalamo-hypophyseal system controlling diuresis through the action of antidiuretic hormone on the kidney is well established, some doubt exists of the presence of a hypothalamic center controlling drinking *per se.* Many investigators believe that thirst is a non-specific reaction to alterations in the electrolyte composition of body fluids. Brobeck and his collaborators have reported the existence of a primary "eating center" in the lateral hypothalamus(1,2). Destruction of this area will cause animals to stop eating and die of starvation while direct stimulation will cause them to eat voraciously. A similar center controlling drinking has not yet been described, however.

In the course of investigations on the effect of hypothalamic stimulation on the endocrine system of albino rats, it was noted that in one

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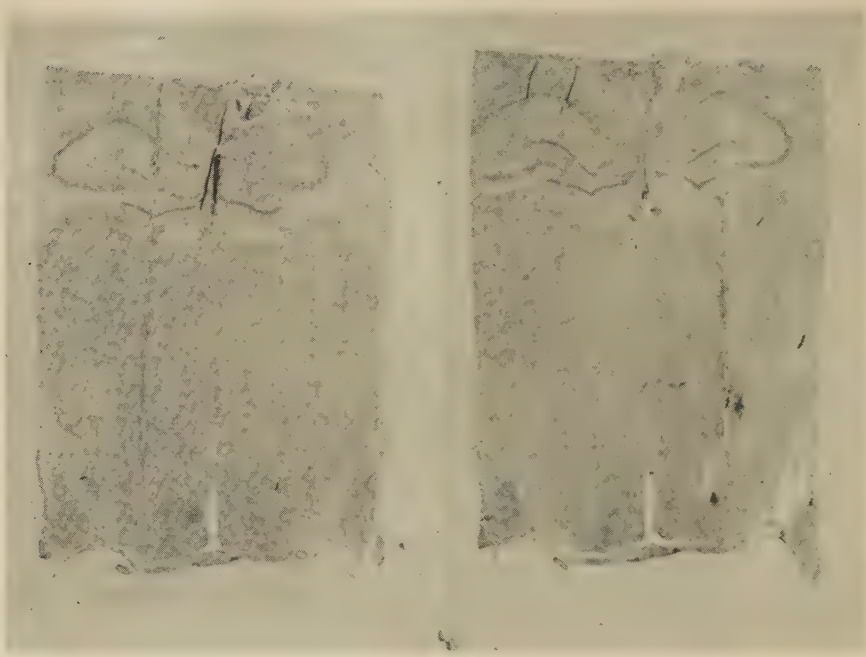

FIG. 1a. Needle track of right electrode showing termination just above ventromedian nucleus.
FIG. 1b. Needle track of left electrode.

animal the electrodes were apparently placed in a center which did have primary control over drinking behavior. Although only one animal to date out of 25 stimulated in various hypothalamic areas has yet shown this reaction, the results in this one animal were so clear cut and so striking that it is felt a preliminary report on this phenomenon may be of interest.

Materials and methods. A system of stimulating the central nervous system of rats by radiofrequency activation of a unit buried subcutaneously has been devised and will be described in a later communication. The system is quite similar to that used by Hume(3). In brief, 2 platinum-iridium electrodes, 0.3 mm in diameter, 8.5 mm long, and 3 mm apart were imbedded in a lucite-silver unit and placed in the hypothalamus of a 259 g female Holtzman rat with a stereotaxic instrument. The unit was fixed tightly to the skull with silver screws and leads from the electrodes went to a non-directional pickup loop which was placed subcutaneously between the scapulae. The electrodes were insulated with 3 coats of lucite except for the tips, which were ground to 60° points. Stimulation was carried out by means of a 60 cycle/sec. square wave DC pulse of 2 milliseconds duration. Except for periods of study of reaction to acute changes in stimulation, the impulses were controlled by a timer which gave 5 minutes of stimulation followed by 25 minutes of rest. The exact current flowing across the electrodes could not be determined, but from studies with other rats of the same group it was calculated that the animal began reacting when 0.5-1 volt was developed across the electrodes and began having convulsions when 5-8 volts were developed. The impedance of the rat was calculated as 5-10,000 ohms. At *autopsy*, all endocrine organs were weighed. The unit was removed with the stereotaxic instrument and the skull trimmed of skin and excess tissue, a large section of the dorsum of the skull removed, and the remainder of the head was fixed in sublimate-formol for one week. Serial sections at 10 μ were then made through the hypothalamic area, every tenth section mounted, and the sections stained with toluidine blue for 6 hours.

Results. Stimulation of the animal began

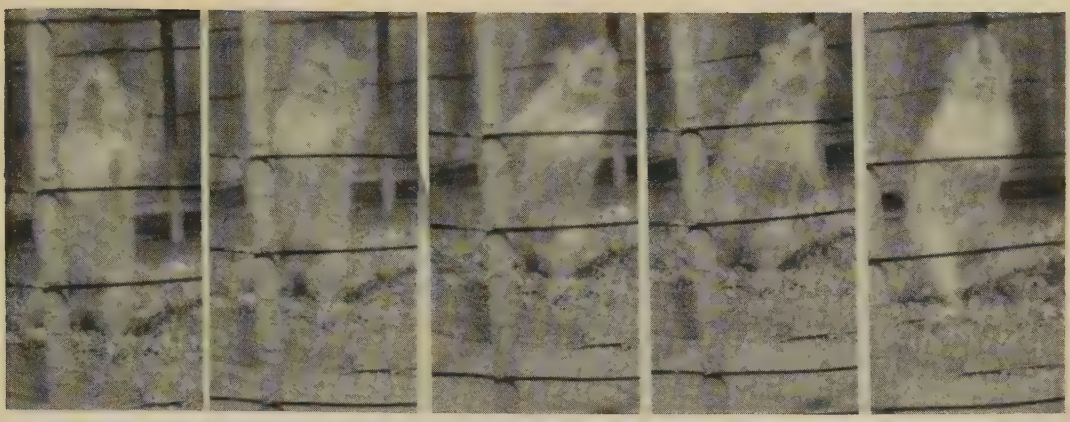

FIG. 2. Consecutive frames from motion picture of rat during first day of stimulation. Activity at this time was limited to violent licking during stimulation. Animal is shown licking the glass wall of the cage.

24 hours after the electrodes were implanted. It was immediately apparent that the animal was under great compulsion to perform violent "licking" activity when a current was passed between the hypothalamic electrodes. In response to stimulation, it would stand on its hind legs and run vigorously around the glass enclosed circular cage, licking wildly at the glass wall. This behaviour would cease immediately upon shutting off the current. If the voltage were slowly increased, licking would gradually become more vigorous.

With stimulation continuing by timer control, the reaction of the animal changed during the first night. The water bottle containing 200 ml was found completely empty at 9 a. m. even though it had been filled at 6 p. m. the previous evening. It was now found that stimulation would result in violent drinking activity. The non-specific licking response had been lost. As soon as the current was turned on, the animal would jump for the water bottle and continue to drink avidly until the switch was turned off. If the water bottle were removed and the current then turned on, the rat would go back to its "licking" behaviour of the previous day, but would immediately transfer it to drinking behaviour when the water bottle was replaced.

If the voltage were slowly turned up, the animal would gradually lose its drinking behaviour and become hyperactive and squeal, but it would still transform part of this stimulation into drinking. When the tap water in the drinking bottle was replaced with 5% saline, the rat would begin by drinking as vigorously as before, but it was obvious that the saline was quite distasteful. She would push at the water spout with her forepaws and would finally be able to force herself away from the bottle for a few seconds, then the compulsion to drink would again become too great and she would be forced back to lapping at the saline. It was possible by changing the strength of the current to produce a response that would range from only partial drinking activity when tap water was used, through violent and sustained drinking activity, to hyperreactivity, jumping and convulsions. During this period of marked drinking behaviour the rat drank in excess of 400 ml tap water daily. It was being chronically stimulated 5 minutes every half hour.

By the fourth day of stimulation, the rat had begun to lose its response to stimulation. It would still respond by drinking, but the voltage of the transmitter had to be turned much higher than previously. During the fourth day its water consumption fell to about 50 ml. On the fifth day the animal no longer responded to stimulation, even though the transmitter was operating at maximum strength. It was then killed and autopsied. The weights of the endocrine glands were within normal limits. (Table I).

On sectioning, it was found that the tips of the electrodes were situated immediately behind the level of the paraventricular nuclei, at

TABLE I. Body and Endocrine Weights at Autopsy.

Body wt, g	Thyroid	Ovaries	Adrenals	Uterus
	mg			
254	13.9	70.8	78.5	337.3

the lateral edge of the dorso-median nuclei and in the area between the dorso-median and ventro-median nuclei. Although the needle tracks were easily visible there was no obvious reaction around the electrodes. The remainder of the hypothalamus did not appear abnormal. Both the paraventricular and supraoptic nuclei appeared intact and contained a normal complement of the typical large cells.

Discussion. Although this report is limited to one animal, the results are so striking that it is difficult to postulate any other mechanism than that a "drinking center" was being directly stimulated. This center seems to be located in approximately the same area as the "eating center," but lying somewhat anterior.

It is extremely unlikely that the drinking behaviour observed was due to inhibition or stimulation of the hypothalamo-hypophyseal pathways controlling antidiuretic hormone release. The drinking response to stimulation was immediate while if it had been secondary to diuresis one would expect a considerable lag period. A true integrated behavioural mechanism would seem to be involved. Hess and his coworkers have previously obtained complex autonomic actions from hypothalamic stimulation, but so far as can be learned, drinking behaviour of this type has never been observed in their experiments. Only nonpurposeful licking movements have been obtained.

Andersson has previously reported suggestive evidence of the existence of a "thirst center." He made stereotaxically controlled injections of hypertonic saline into the hypothalamus of goats and obtained a marked drinking response(4). The area in which he obtained a positive reaction appears to roughly correspond to the area herein reported.

The loss of reaction by the fifth day of stimulation seems to be characteristic of the stimulating system as presently employed. Although the animal reported is the only one which has shown drinking behaviour, all other stimulated animals have shown marked reactions to the current. These are usually characterized by hyperactivity, rage, squealing, and, if the stimulation is great enough, convulsions. In all cases, all response is lost after a few days of chronic stimulation. The exact cause of the loss of response is not known, but the most likely explanation is that polarization occurs at the electrodes. It has been noted in animals in which the impedance across the hypothalamic electrodes has been measured that the impedance increases 10-20 fold in a few days and that this increase corresponds with a decreased responsiveness of the animal to stimulation.

Summary. In a female rat with 2 bipolar bilateral electrodes in the lateral hypothalamus at the level of the lower dorsomedian nucleus, violent drinking activity could be stimulated by passing a 60 cycle, 2 msec. D.C. pulse across the electrodes by RF activation of a subcutaneously buried pickup unit. During the period of maximal response, the rat drank in excess of 400 ml tap water daily while chronically stimulated. By the fifth day the animal had lost its responsiveness to stimulation. The drinking behaviour was not secondary to diuresis but was an immediate and direct response to hypothalamic stimulation.

I am indebted to Mr. Howard Erwin for valuable technical assistance.

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Comparative Absorption of Vitamin B_{12} Analogues by Normal Humans. I. Chlorocobalamin vs. Cyanocobalamin. (21717)

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Recent developments in the field of vit. B_{12} chemistry have revealed the existence of a whole host of compounds resembling vit. B_{12} in basic structure or directly derivable therefrom. The simplest group of these compounds is the analogous cobalamins(1-6) which differ from cyanocobalamin by the substitution of a water molecule or an anionic group for the cyanide. These analogues differ markedly in the strength of the cobalamin-substituent binding. Thus the cyano group in cyanocobalamin is so tightly bound that vit. B_{12} is essentially a non-electrolyte. By contrast, analogues such as the chlorocobalamin are highly dissociated, by a mechanism(2,4) which may be represented as

$$MCl \xrightarrow{H_2O} [M \cdot H_2O]^+ + Cl^-,$$

so as to be effectively electrolytes in character. In this equation MCl represents "undissociated" chlorocobalamin and $[M \cdot H_2O]^+$ designates the aquocobalamin ion. M is the cobalamin residue, which is representable by $\left[\begin{smallmatrix} -- \\ \\ O\ O\ O \end{smallmatrix} Co^{+++} \right]^+$. This distinction is supported by the relative affinity of cyanocobalamin for organic solvents in measurements of partition coefficients(2,4,6) as contrasted with the water-seeking tendency of chlorocobalamin. It is natural to inquire whether this difference in behavior affects the ease with which the several cobalamins are absorbed from the human gastrointestinal tract. To this end, cobalt 60-labeled vit. B_{12} was converted to the chloro-analog and comparative oral absorption studies performed by the urinary excretion method of Schilling(7). Normal cyano- and chlorocobalamin were injected, for purposes of flushing out absorbed radioactive vitamin, with identical results. The excretion of radioactivity in urine after feeding of labeled chlorocobalamin was much less than that observed in the case of labeled cyanocobalamin. This would indicate the marked superiority of cyanocobalamin over chlorocobalamin for absorption by normal humans.

Experimental. Materials. The vit. B_{12}-Co^{60} employed in these experiments had a specific activity of approximately 200 μc/mg. Its preparation and purity have been described elsewhere(8,9). The conversion of the radioactive cyanocobalamin to the chloro-analog was accomplished by the photochemical method described by Boxer and Rickards (10), and by Veer, Edelhausen, Wijmenga, and Lens(11). A 0.02 N HCl solution with a cyanocobalamin concentration of $\cong$ 70 μg per ml was irradiated for 26 hours with continuous aspiration by a stream of nitrogen. Liberated cyanide was collected in alkali and determined colorimetrically(12) to insure quantitative cyanide removal.* The labeled chlorocobalamin was removed from the acid solution by extraction with a 1/1 cresol-carbon tetrachloride solution and precipitation by addition of acetone and ether. The solid chlorocobalamin was filtered and dissolved in water to yield the stock solution (66.6 μg/ml) diluted to 1 μg/ml for oral administration (13). The concentration of this solution was verified by microbial assay with *L. leichmannii* according to the direction of Skeggs *et al.*(14). The shape of the absorption spectrum determined with a Cary recording spectrophotometer of this product in deionized water, and the distribution between water and benzyl alcohol, were identical with that of non-radioactive material. The following absorptivities were determined for chlorocobalamin in de-

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TABLE I. Urinary Excretion (in % of 2 μg Oral Dose ± Standard Deviation) of Radioactivity after Injection.

			Group	I	II	III	IV
Treatment	Oral labeled cobalamin			chloro	chloro	cyano	cyano
	Inj. normal cobalamin			"	cyano	"	chloro
Avg % of dose excreted ± stand. dev.	First study	No. subjects		5	5	8	—
		24 hr excretion		3.0 ± .7	2.7 ± .6	11.3 ± 4.2	—
	Second study	No. subjects		5	5	6	5
		24 hr excretion		2.5 ± .7	2.5 ± .7	13.6 ± 2.0	13.6 ± 5.9
		24-28 hr excretion		.3 ± .1	.2 ± .06	.2 ± .1	1.8 ± .9

ionized water and in dilute HCl solution: λ_{max} 273 mμ, $E^{1\%}_{1\,cm}$ 155; λ_{max} 351 mμ, $E^{1\%}_{1\,cm}$ 195; λ_{max} 409 mμ, $E^{1\%}_{1\,cm}$ 29; λ_{max} 498 mμ, $E^{1\%}_{1\,cm}$ 62; λ_{max} 525 mμ, $E^{1\%}_{1\,cm}$ 66. The cyanocobalamin used for injection was Merck crystalline vit. B_{12}. The chlorocobalamin was also prepared by the photochemical method cited above and found to possess the characteristic absorption spectrum and correct chlorine content for this compound. Injection solutions contained 1 mg of cobalamin/1 ml of physiological saline.

Method. The Schilling method(7) was employed to estimate the extent of absorption of the labeled cobalamins. Oral administration of 2 μg of labeled chloro- or cyanocobalamin was followed in 2 hours by subcutaneous injection of 1 ml of injection solution, and urines collected for a period of 24 hours for radioactivity measurement. In certain cases, a second 24-hour urine collection was made. Radioactivity measurements were made by scintillation counting of urine samples after concentration by evaporation(13). Under these conditions, the 2 μg dose gave an activity of approximately 25,000 counts per minute. Two studies were performed at different times and with different groups of young normal adult males of mean age 30.2 years (range 21-40). Continual supervision of these individuals was provided, to insure complete urine collections. Subjects were divided into 3 (first study) and 4 groups (second study) who received the following treatment: *Group I*—Received orally 2 μg of labeled chlorocobalamin; followed by injection of 1 mg of normal chlorocobalamin. *Group II*—Received orally 2 μg of labeled chlorocobalamin; followed by injection of 1 mg of normal cyanocobalamin. *Group III*—Received orally 2 μg of labeled cyanocobalamin; followed by injection of 1 mg of normal cyanocobalamin. *Group IV*—Received orally 2 μg of labeled cyanocobalamine; followed by injection of 1 mg of normal chlorocobalamin.

Results. The urinary radioactivities observed in these studies are summarized below in Table I. These responses are expressed in terms of percent of oral dose of radioactive vitamin which was excreted in the urine during the collection period specified. This computation assumes that the radioactivity flushed into the urine as a result of injection is actually in its original oral form, which appears to be true at least in the case of oral cyanocobalamin(15).

Responses reported in Table I are average percentage excretions for an entire group of subjects, together with standard deviations. The number of subjects in a given group is also shown. Between these two studies, a total of 10 or more subjects were involved in each group except Group IV which consisted of 5 persons.

Discussion. It appears from Table I that the average urinary output of radioactivity by subjects in Groups I and II receiving 2 μg of labeled chlorocobalamin is only 2.5-3.0% as compared to the 11.3-13.6% excretion ob-

served with Groups III and IV fed 2 μg of labeled cyanocobalamin. Group III is in reality a control group since these individuals were treated by the standard procedure of Schilling. Although normal individuals exhibit wide variations in their responses to this test, the overall average urinary excretion of radioactivity is approximately 12%(13) as found in Groups III and IV. One may conclude from this disparity that the absorption of oral chlorocobalamin by the human is only a fraction of that of vit. B_{12} (cyanocobalamin) when both are fed in 2 μg doses.

This difference is not due to a slower rate of excretion of the chloro analog since the additional 0.3% and 0.2% radioactivity excreted by Groups I and II during the second 24-hour period is but slight, and comparable to the behavior of cyanocobalamin (Groups III and IV) in this respect.

Of interest is the fact that chlorocobalamin and cyanocobalamin are indistinguishable as regards their ability to liberate the labeled cobalamins upon injection. Thus Groups I and II which received injections of massive doses of normal chloro- and cyanocobalamin respectively yield essentially identical results with oral chlorocobalamin. Similarly, excretion of labeled cyanocobalamin by subjects of Groups III and IV was equal despite the difference in the nature of the injected cobalamin. Apparently injected cobalamins are equivalent one to another, or are converted in tissue to a common form; and the same must be true for the oral cobalamins once absorbed. It may be that parenterally administered cobalamins are equally effective in the treatment of pernicious anemia. This equivalence does not apply, however, to the ingestion of cobalamins, which obviously differ markedly in their ease of absorption.

One is tempted to attribute the disparate ease of absorption or tissue retention of these cobalamins upon oral administration to the difference in their electrolyte properties. Cyanocobalamin, with its tightly bound cyanide group, is effectively an organic molecule with a relative preference for solution in organic solvents. Chlorocobalamin, on the other hand, exists in neutral or acid medium, as ionized aquocobalamin chloride, with a marked tendency for solution in aqueous media. If we consider intestinal absorption of cobalamins to involve, at least at one stage, a partition between the aqueous intestinal contents and the organic intestinal mucosa, then the dissimilar behavior of the two cobalamins studied appears logical.

On this basis, hydroxocobalamin would behave identically with chlorocobalamin in the normal human since the hydroxo-analog would be converted to chlorocobalamin at the acidity of normal gastric juice. Other anion analogs may not be so dependent upon pH. A number of such compounds varying in ease of dissociation are to be studied by the method here described to test the hypothesis proposed.

The lower absorption reported here for chlorocobalamin is supported by lower B_{12} blood level changes noted after oral administration of massive doses of normal chlorocobalamin than after similar administration of cyanocobalamin(16).

Summary. 1. Urinary excretion tests performed with cobalt-60 labeled cobalamins by the method of Schilling indicate that the absorption of chlorocobalamin at the 2 μg dose level by the normal young adult male is much less than that of cyanocobalamin at the same oral level. 2. Normal chlorocobalamin and cyanocobalamin appear to be equivalent in their capacity for liberating absorbed labeled cobalamins by injection of massive doses. 3. Differences in the ease of absorption of the cobalamins are attributed to differences in their electrolyte nature and attendant solubility properties.

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Tryptose Phosphate Broth as Supplementary Factor for Maintenance of HeLa Cell Tissue Cultures.* (21718)

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Prolonged maintenance of HeLa cells(1) in tissue culture in the absence of human serum has been difficult. Indeed, it has been obvious that certain factors essential for maintenance and growth of these cells in tissue culture were not contained in the chemically defined maintenance solution-chicken serum mixture recommended by Scherer, Syverton, and Gey(1). During the investigation of an epidemic of acute non-bacterial pharyngitis(2), it was observed that throat washings prepared in nutrient broths for the isolation of viruses in HeLa cell tissue cultures not only maintained these cells in good condition but also appeared to stimulate their multiplication. On the other hand, pharyngeal secretions obtained in Hanks' balanced salt solution had no such salutary effect upon HeLa cell cultures. This observation suggested that bacteriological nutrient broths may contain one or more factors necessary for maintenance of cells' health and growth. Carrel and Baker demonstrated the value of certain peptones for maintenance and growth of chicken fibroblasts(3). The observation that nutrient broths appeared to contain an effective auxiliary factor or factors for maintenance of HeLa cells seemed to be consistent with these earlier findings(3). Studies were carried out to determine the ability of various bacteriological broths containing peptones to assist in maintenance of HeLa cell cultures. It is the purpose of this paper to present evidence that tryptose phosphate broth mixed with maintenance solution (termed MS) and chicken serum was the most effective broth tested; that this solution not only maintained HeLa cells in good condition but also supported multiplication of these cells; that infectivity titers of the RI-67 virus (termed ARD virus(4)) isolated by Hilleman and Werner(5), the Adenoid Degeneration (AD) agents isolated by Rowe *et al.*(6), and Type I poliomyelitis virus were significantly increased when measured with HeLa cells in the presence of tryptose phosphate broth; and that multiplication of the ARD virus was increased when tryptose phosphate broth was mixed with MS and chicken serum.

Materials and methods. Tissue culture. Strain HeLa cells (Gey) originally derived from an epidermoid carcinoma of the uterus

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were grown serially in tissue culture by a modification of the methods described by Scherer, Syverton, and Gey(1). Cells were grown directly on glass in 32-oz. duraglass prescription bottles. The nutrient fluid employed consisted of 40% human serum and 60% balanced Hanks' solution. For infectivity titrations, HeLa cells were grown in screw-top tubes, 50,000 cells per tube, in the same nutrient fluid. To prepare cultures for tubes or bottles, cell suspensions were made by mechanical means; trypsin was not used. Details of the methods followed have been described elsewhere(4). *Viruses.* The viruses employed were: RI-67 strain of ARD virus(5) kindly supplied by Dr. M. R. Hilleman; Types I, II, and III of the Adenoid Degeneration or "AD" agents(6) (recently called the adenoidal-pharyngeal-conjunctival viruses(7)) furnished by Dr. R. J. Huebner; and the Mahoney strain of Type I poliomyelitis virus. Viruses were grown in HeLa cell cultures washed free of human serum, to which was added a maintenance mixture consisting of Scherer's MS 90%(1) and chicken serum 10%, or MS 67.5%, chicken serum 7.5% and tryptose phosphate broth (Difco) 25%. Infected cultures were incubated at 36°C until complete cytopathogenic effects were observed. Pools of ARD and AD agents were prepared by freezing and thawing the infected cell suspensions 6 times, and removing cellular debris by centrifugation at 2500 RPM for 10 minutes. Cell disruption was not essential for preparation of poliomyelitis virus pools. *Virus infectivity titrations.* Serial 1:3.2 ($10^{-0.5}$) dilutions of infected tissue culture homogenates were prepared in Hanks' balanced salt solution. To each of 2 HeLa cell tissue culture tubes 0.1 ml of virus dilution was added, and the tubes were incubated at 36°C for 6 days. A culture was considered infected when 50% or more of the HeLa cells had undergone specific cytopathogenic changes. The endpoint was calculated as the highest dilution of virus which infected one-half of the tubes (8). *Cell counts.* To enumerate the number of cells in tissue culture tubes, uniform cell suspensions were prepared by use of the chelating agent, Versene acid (ethylene diamine tetra acetic acid).‡ Tissue culture fluid was removed, and 0.5 ml of versene acid (referred to as Versene), 0.05% by weight in 0.85% sodium chloride was added. Cells were scraped from the wall of the tube by means of a rubber policeman on a glass rod, and the cell suspension prepared by pipetting 15 to 20 times with a 1-ml pipette. Cells were then counted in a hemocytometer. For comparative cell counts, 3 tubes were employed for every value; each cell suspension was prepared and enumerated individually, and the geometric mean of the 3 counts computed.

Results. Effect of various broths on maintenance of HeLa cell cultures. Extensive multiplication of HeLa cells in tissue culture requires human serum(1,9). For infection of these cells with viruses, however, human serum must be removed to free cells from antibodies, and the cells must be maintained in the absence of human serum. A chemically defined mixture termed "maintenance solution" (MS), to which chicken serum to a volume of 10% is added, has been recommended(1). This mixture, however, does not permit maintenance of cultures longer than a week without frequent changes of fluid. Despite the addition of fresh fluid at frequent intervals, HeLa cells decrease in number, become granular, and non-specific rounding and clumping of cells may occur.

The observation that the addition of nutrient broth to the customary infecting fluid not only maintained the healthy appearance of cells but also the continuity of cellular sheets, suggested the routine use of broth in culture fluids. To investigate this possibility a number of bacteriological broth media were tested to determine their effect on maintenance of HeLa cells in culture. The original mixture of maintenance solution containing 10% chicken serum was considered the standard of reference, and the efficacy of all other solutions was compared with this mixture. Comparisons were based upon appearance of cells, estimation of number of cells, and maintenance of a solid sheet of cells. The results of these studies, summarized in Table I, indicate that tryptose phosphate broth was the

‡ Obtained from Versenes, Inc., Framingham, Mass.

TABLE I. Effect of Various Bacteriological Broths on Maintenance of HeLa Cell Cultures as Compared to Standard Solution (MS 90 + CS 10).

Maintenance mixture	Relative effect on cells: Number	Relative effect on cells: Appearance¶
MS* 90 + CS† 10	0‡	Cells granular and decreased in number
" 67.5 + " 7.5 + Tryptose phosphate broth 25	++++§	Solid sheet of cells; cells least granular
Idem + " broth 25	+++	Cells granular; good sheet of cells
" + Brain-heart infusion 25	+++	Many round cells; good sheet of cells
" + Trypticase soy broth 25	+++	Cells very granular; good sheet of cells
" + Beef infusion broth 25	+	Many round cells; granular cells; no solid sheet of cells
" + Glucose peptone yeast broth 25	—‖	Cells rounded and fell off glass
" + Nutrient broth 25	++++	" granular; good sheet of cells
Hanks' 63 + CS 7 + Tryptose phosphate broth 30	++	" " ; no sheet of cells
Tryptose phosphate broth	—	" died rapidly

* MS = Scherer's maintenance solution(1). † CS = Chicken serum. ‡ Standard of reference. § No. of +'s refers to relative increase in No. of cells. ‖ Poorer than stand. of reference. ¶ Observed daily for 7 days.

optimum supplement tested. Although nutrient broth added to maintenance solution and chicken serum appeared to stimulate cell multiplication and persistence of a solid sheet of cells, the cytoplasm of the HeLa cells was considerably more granular than in cultures in which the maintenance mixture contained tryptose phosphate broth. Subsequent experiments showed that HeLa cells could be maintained in excellent condition for 7 to 10 days without addition or change of maintenance mixture. Moreover, the cells appeared to be in good condition for at least 2 weeks if the maintenance fluid was changed, or the pH of the original culture fluid was neutralized and 0.3 ml of new solution containing 25% tryptose phosphate broth was added after 7 days. With more frequent additions or changes of the culture fluid, HeLa cells were maintained for even more prolonged periods.

Effect of tryptose phosphate broth on HeLa cell multiplication. HeLa cells appeared to multiply in cultures in which tryptose phosphate broth was added to the customary maintenance fluid. To determine whether or not multiplication could be stimulated by tryptose phosphate broth, HeLa cell cultures were prepared, washed free of human serum, and maintenance fluids with and without broth as a supplement were added. Direct cell counts were made on 3 separate tubes for each solution tested at every time interval. The data from a typical experiment are summarized in Fig. 1. Whereas MS and chicken serum maintained cell cultures for 4 days, there was no

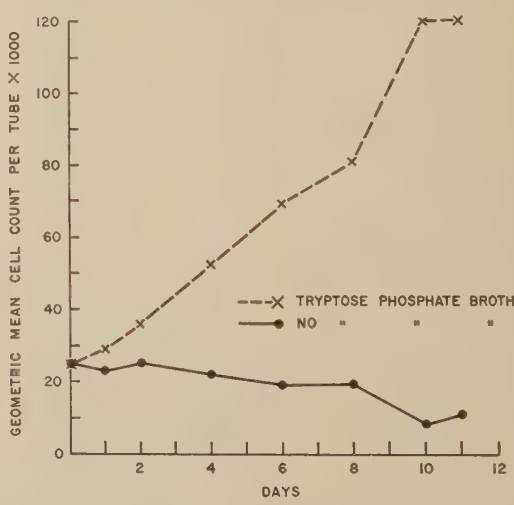

FIG. 1. Comparison of cell counts of HeLa cell cultures sustained in maintenance mixtures containing either tryptose phosphate broth 25%, MS 67.5%, and chicken serum 7.5%, or MS 92.5% and chicken serum 7.5%. Each value plotted represents the geometric mean of cell counts carried out on 3 cultures.

TABLE II. Comparison of Infectivity Titers of Viruses Determined in Tissue Cultures Maintained with and without Tryptose Phosphate Broth (TPB).

Virus	Infectivity titer (log) — Maintenance mixture: MS + CS*	Infectivity titer (log) — Maintenance mixture: MS + CS + TPB†
ARD	−2.75	−4.00
AD I	−2.75	−3.75
II	−2.75	−3.50
III	−2.50	−3.50
Poliomyelitis, Type I	−4.25	−5.25

* Scherer's maintenance solution 92.5% and chicken serum 7.5%.

† Scherer's maintenance solution 67.5% and chicken serum 7.5% and tryptose phosphate broth 25%.

increase in the number of cells, and indeed, after 4 days there was a decrease in the quantity of cells present. In contrast, tryptose phosphate broth, when added to MS and chicken serum, supported continued cell multiplication for at least 11 days. The conclusion that tryptose phosphate broth contains a factor important for maintenance of cells was supported by evidence obtained from experiments which demonstrated that tubes of HeLa cell cultures could be prepared from bottles by suspending the cells directly in the broth, MS and chicken serum mixture. The only human serum present in these cultures was the very small quantity in the unwashed HeLa cell suspension. Successful HeLa cell tube cultures could not be made when tryptose phosphate broth was not included in the MS-chicken serum mixture.

Effect of tryptose phosphate broth on viral multiplication in HeLa cells. Inclusion of a new substance in the media of cell cultures employed for the investigation of viruses required that the effect of this substance on viral propagation be tested. Infectivity titrations of the ARD and AD agents, as well as Type I poliomyelitis virus, were carried out in HeLa cell cultures prepared at the same time and maintained with or without tryptose phosphate broth in the maintenance mixture. The results of these parallel titrations, summarized in Table II, indicate that in the HeLa cell cultures containing tryptose phosphate broth an infectivity titer of 1.0 log or greater was obtained with not only the new respiratory agents but also with Type I poliomyelitis virus. One cannot deduce from these data whether the broth increased the sensitivity of HeLa cells to the cytopathogenic effects of these agents, or whether cells maintained in this culture media supported the multiplication of a larger quantity of virus.

To investigate the action of tryptose phosphate broth in these experiments, the exact amount of virus synthesized in HeLa cell cultures maintained with and without broth was determined. For this purpose 4 ml of a $10^{-1.0}$ dilution of ARD virus was inoculated into cultures of HeLa cells grown in duraglass bottles. Infected cells were harvested and virus pools prepared from both sets of bottles 4 days after infection when maximum viral cytopathogenic effects had occurred in each. The results of infectivity titrations carried out with the 2 pools of virus are presented in Table III. Only 10% as much ARD virus was propagated in cells maintained with MS and chicken serum as in cultures to which tryptose phosphate broth to a 25% volume was added. These data suggest that the higher infectivity titer obtained with tryptose phosphate broth in the maintenance solution was a reflection of increased viral multiplication in these cultures.

Optimum concentration of tryptose phosphate broth for infectivity titrations. Experiments were next carried out to determine the concentration of tryptose phosphate broth in maintenance media which would yield maximum infectivity titers. Infectivity titrations with a single series of 0.5 log dilutions of ARD virus were done in HeLa cell cultures maintained with media containing MS, 7.5% chicken serum and concentrations of tryptose phosphate broth varying from 0 to 25%. The results of a typical experiment, presented in

TABLE III. Comparison of Multiplication of ARD Virus in HeLa Cell Cultures Maintained with and without Tryptose Phosphate Broth (TPB).

Maintenance mixture	Infectivity titer of ARD virus infected cultures (log)
MS + CS	−2.75
MS + CS + TPB	−3.75

TABLE IV. Concentration of Tryptose Phosphate Broth Required to Obtain Maximum Infectivity Titer of ARD Virus.

Concentration tryptose phosphate broth	Infectivity titer ARD virus
(%)	(log)
0	−2.75
5	−3.75
10	−3.75
15	−4.25
20	−4.00
25	−4.25

Table IV, indicate that at least a 15% concentration of tryptose phosphate broth was required to detect the smallest amount of virus by infectivity titrations. As little as 5% broth, however, produced a significant increase in titer of ARD virus.

Discussion. The evidence presented indicates that tryptose phosphate broth acts as a supplementary growth factor for HeLa cells in tissue culture. This relatively simple broth not only permitted maintenance of HeLa cell cultures for prolonged periods in the absence of human serum, but also stimulated multiplication of these cells. Moreover, HeLa cells maintained with MS, chicken serum and tryptose phosphate broth in a concentration of 15% or greater permitted the detection of considerably smaller quantities of ARD, AD and Type I poliomyelitis viruses than could be measured without the addition of broth. Increased propagation of virus in cells maintained with tryptose phosphate broth as a supplement, as demonstrated with ARD virus, was probably an important factor in this phenomenon. The increased quantity of virus produced in these cultures may have been accomplished by: 1) maintenance of host cells in a better metabolic state; 2) addition of material which could be utilized directly for increased viral synthesis; or 3) an increased number of host cells. Capacity to detect smaller quantities of virus may also result from an increased sensitivity of HeLa cells to viral cytopathogenic effects. Preliminary data indicate that the detection of smaller quantities of virus in HeLa cells maintained in the presence of tryptose phosphate broth may also permit more ready isolation of virus from patients.

In addition to the practical applications for the use of tryptose phosphate broth in studies of viruses in HeLa cell tissue cultures, certain theoretical implications may be considered. That some factor or factors in tryptose phosphate broth are important growth supplements for HeLa cells is evident. The isolation and identification of substances which are essential or supplementary for cell multiplication and growth would be of considerable importance for detailed studies of metabolism, growth, and development of normal and abnormal cells. Moreover, identification of the substance in tryptose phosphate broth which permitted the host cell to synthesize an increased quantity of virus may add to knowledge relative to factors essential for viral replication.

Baker and Carrel demonstrated that the proteose present in Witte's peptone was an ingredient which stimulated growth of chick embryo fibroblasts in the absence of serum (3,10). Studies are in progress to attempt to identify and isolate from tryptose phosphate broth the factor or factors present which are responsible for the phenomena observed. Preliminary data indicate that proteoses present in tryptose phosphate broth are active in supporting the growth of HeLa cells(11).

Summary. A mixture of tryptose phosphate broth, 15 to 25%, maintenance solution (MS), 67.5 to 77.5%, and chicken serum, 7.5%, maintained HeLa cells in tissue culture for at least 10 days. During this period HeLa cells increased 3 to 5 fold in number. Furthermore, smaller quantities of ARD, AD and Type I poliomyelitis viruses could be detected and significantly more ARD virus could be propagated in HeLa cells maintained in this medium than in cells supported with maintenance solution and chicken serum alone.

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Nutrition of Animal Cells in Tissue Culture. X. Synthetic Medium No. 858.*† (21719)

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A previous report from this laboratory(1) described a chemically defined medium, solution No. 703, that contained 58 ingredients and was capable of yielding, in one week, 5- and 6-fold increases in the population of replicate cultures prepared from washed suspensions of Earle's L strain cells from the mouse. The medium consisted of certain amino acids, vitamins, lipid sources, coenzymes, intermediary metabolites, and antibiotics, in a balanced saline solution containing glucose. Over the past year, new media have been devised that are capable of yielding 9- and 10-fold increases in the population of replicate cultures of L strain cells, in one week. It is the purpose of the present communication to describe the steps that were taken to achieve this improvement and to report the composition of the most adequate medium thus far devised, solution No. 858.

Materials and methods. All quantitative assays reported here were made with Earle's L strain cells from the mouse(2). The media used were *natural* (consisting of 40% horse serum, 3% chick embryo extract, and 0.002% phenol red, in a balanced saline solution containing glucose), *synthetic* (chemically defined), and synthetic supplemented with natural body substances. The synthetic media were solutions 635 and 703(1), and modifications of them. The quantitative procedures followed in the preparation of replicate cell cultures were originally developed by Earle and his associates(3,4) and modified by us(5) for use with synthetic media. In brief, washed cell suspensions were prepared in solution 635 from L strain cultures propagated for 3-4 days in large Kolle flasks in 25 ml of natural medium or, more recently, in solution 858 supplemented with 20% horse serum. After the washed cell suspensions were screened to eliminate cell clumps, they were transferred to a reservoir assembly in which they were stirred mechanically and dispensed, eventually, as replicate 0.5-ml samples into a series of 20-24 T-15 culture flasks to which the medium (2.0 ml) had already been added. In experiments in which two media were to be compared, each was placed in alternate flasks. The number of cells placed in each culture of a series was estimated by counting the cell nuclei in representative samples of the stirred inoculum. The samples were prepared for counting by treating the cells with citric acid followed by low speed centrifugation and light staining with crystal violet. The liberated,

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FIG. 1-6. Living cells from 3 different strains in D-3.5 Carrel flasks after various periods of cultivation in synthetic media. 120 ×.

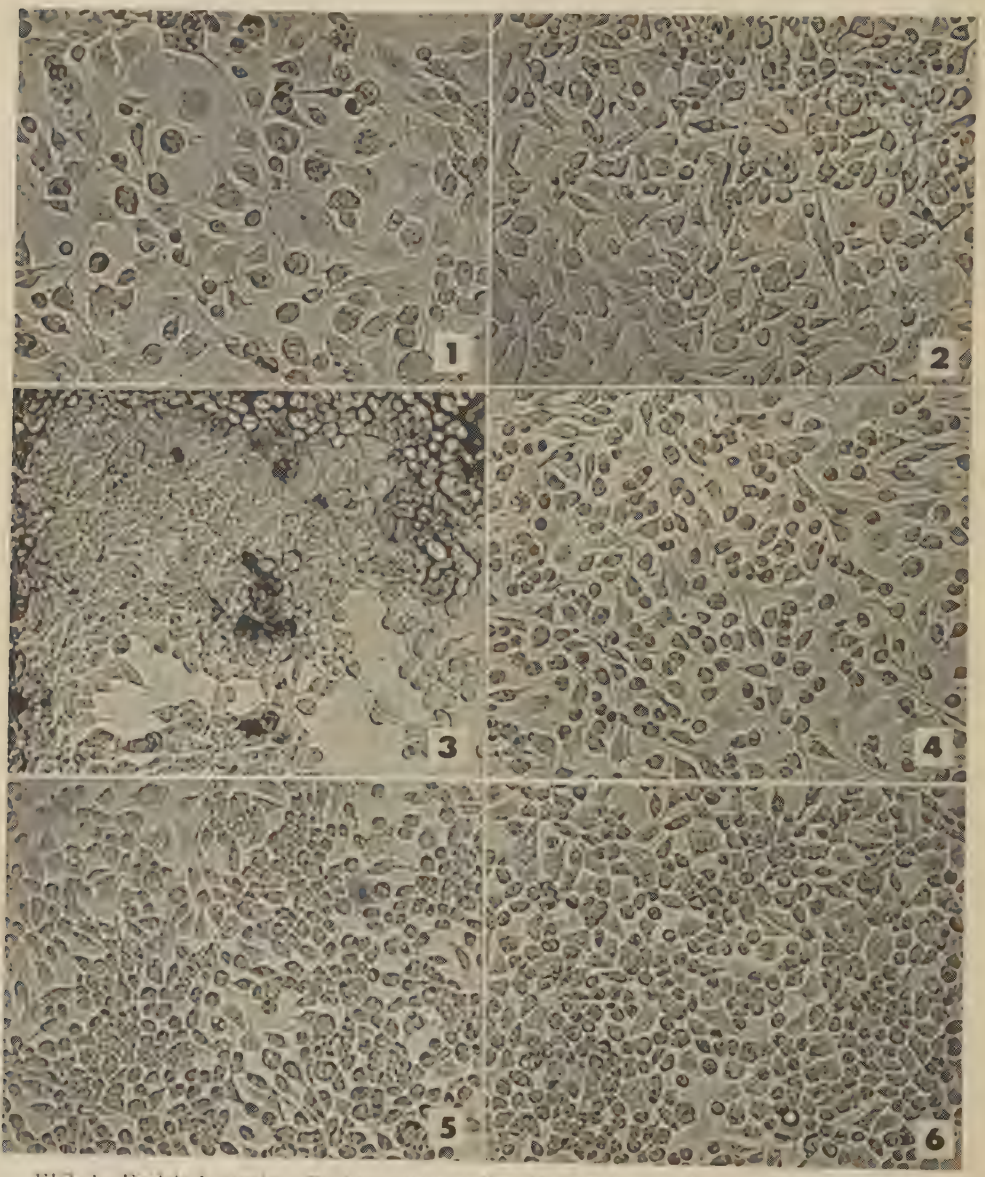

FIG. 1. Earle's L strain cells from mouse after 10 days in solution 703 supplemented with 5 desoxynucleotides and 4 desoxynucleosides (5-methyldesoxycytidine not included), at 0.1 μg/ml each.

FIG. 2. L strain cells from same washed suspension after 10 days in solution 703 supplemented with the same desoxynucleotides and desoxynucleosides, at 10 μg/ml each.

FIG. 3. L strain cells after 343 days in solution 703 supplemented with 0.1% crude chondroitin sulfuric acid.

FIG. 4. L strain cells after 20 days in solution 858.

FIG. 5. Gey's 14p strain cells from rat(21) after 20 days in solution 858.

FIG. 6. McCulloch's MCN strain cells from human bone marrow(22) after 20 days in solution 858.

TABLE I. Synthetic Medium No. 858.

	mg/1000 ml		mg/1000 ml
L-Arginine	70	Diphosphopyridine nucleotide (95% pure)	7
L-Histidine	20	Triphosphopyridine nucleotide (80% pure)	1
L-Lysine	70	Coenzyme A (75% pure)	2.5
L-Tyrosine	40	Cocarboxylase (88% pure)	1
L-Tryptophane	10	Flavin adenine dinucleotide (60% pure)	1
L-Phenylalanine	25	Uridine triphosphate (90% pure)	1
L-Cystine	20	Glutathione	10
L-Methionine	15		
L-Serine	25	Adenine desoxyriboside	10
L-Threonine	30	Guanine desoxyriboside	10
L-Leucine	60	Cytosine desoxyriboside	10
L-Isoleucine	20	5-Methyldesoxycytidine	.1
L-Valine	25	Thymidine	10
L-Glutamic acid	75		
L-Aspartic acid	30	Sodium acetate	50
L-Alanine	25	Sodium glucuronate	4.2
L-Proline	40	L-Glutamine	100
L-Hydroxyproline	10	d-Glucose	1000
L-Cysteine	260	Phenol red (pH indicator)	20
Glycine	50	Ethanol (as an initial solvent for fat-soluble constituents)	16
Pyridoxine	.025		
Pyridoxal	.025		
Biotin	.01	NaCl	6800
Folic acid	.01	KCl	400
Choline	.5	$CaCl_2$	200
Inositol	.05	$MgSO_4 \cdot 7H_2O$	200
p-Aminobenzoic acid	.05	$NaH_2PO_4 \cdot H_2O$	140
Vit. A	.1	$NaHCO_3$	2200
Calciferol	.1	Fe, as $Fe(NO_3)_3$	.1
α-Tocopherol phosphate	.01		
Menadione	.01	Sodium penicillin G	1
Ascorbic acid	50	Dihydrostreptomycin sulfate	100
Tween 80 (oleic acid)	5	n-Butyl parahydroxybenzoate	.2
Cholesterol	.2		

stained nuclei so obtained were counted in a hemocytometer. After 7 and 12 days' incubation, the nuclei were freed from the cells of representative cultures comprising the replicate series and counted as before. Fluid renewals were accomplished twice a week by allowing any loose cells to settle to the tip of the culture flasks and by withdrawing and replacing 1 ml of the cell-free medium. On occasions when chondroitin sulfate was present in the medium, the number of cells was estimated by making DNAP determinations(6). A dense precipitate forms when cultures containing chondroitin sulfate are treated with the acids employed in the liberation of nuclei. At *regular intervals,* one or more synthetic solutions were tested in a preliminary manner by means of a simplified version of the quantitative procedures just described. These tests (5), made in Carrel flasks, were designed to compare the effects of various levels of a substance of nutritional interest and to eliminate all toxic combinations of substances before the solutions were assayed by the more elaborate procedures. Ordinarily, the tests were made from the same washed-cell suspensions used in the preparation of replicate T-flask cultures. Sometimes, however, a particular solution was tested simultaneously on washed suspensions prepared from several cell strains. But the cell suspension was always agitated by hand, rather than mechanically, and an ungraduated pipette was used to transfer 1 or 2 drops of the agitated suspension to each culture flask. The effects of the various solutions were judged by examining the cultures at intervals under the microscope (Fig. 1-6). Three cultures were prepared for each solution tested, and all culture fluids were renewed completely twice a week. No effort was made to prevent the loss of suspended cells.

Ingredients of solution 858 (Table I), with

one exception,‡ are obtained commercially and employed without further purification. Aqueous stock solutions are prepared with water passed through a Barnstead still and then through a mixed-bed ion-exchange column(8). All stock solutions except Nos. 1 and 6 are stored at 4°C, without filtration, for periods not exceeding 30 days. A fresh lot of solution 1 is made up each time a new batch of medium is prepared; solution 6 is stored in the frozen state until used. The ingredients of synthetic medium 858 are shown in Table I. The various stock solutions are prepared as follows: *Sol.* 1: To 400-450 ml of water stirred continuously and heated to about 80°C are added the following: phenol red (water soluble) 20 mg; L-arginine monohydrochloride, 70 mg; L-histidine monohydrochloride, 20 mg; L-lysine monohydrochloride, 70 mg; L-tryptophane, 10 mg; L-phenylalanine, 25 mg; L-methionine, 15 mg; L-serine, 25 mg; L-threonine, 30 mg; L-leucine, 60 mg; L-isoleucine, 20 mg; L-valine, 25 mg; L-glutamic acid, 75 mg; L-aspartic acid, 30 mg; L-α-alanine, 25 mg; L-proline, 40 mg; L-hydroxyproline, 10 mg; glycine, 50 mg; L-cysteine hydrochloride, 260 mg; glutathione, 10 mg; ascorbic acid, 50 mg; L-tyrosine, 40 mg; L-cystine, 20 mg; and sodium acetate trihydrate, 81.5 mg. After the solution has cooled to room temperature, the following are added: L-glutamine, 100 mg; dihydrostreptomycin sulfate, 100 mg; adenine desoxyriboside, 10 mg; guanine desoxyriboside, 10 mg; cytosine desoxyriboside, 10 mg; thymidine, 10 mg; sodium glucuronate monohydrate, 4.2 mg. The ingredients of Earle's balanced saline solution(9) are then added, as follows: sodium chloride, 6.8 g; potassium chloride, 0.4 g; calcium chloride, 0.2 g; magnesium sulphate heptahydrate, 0.2 g; sodium dihydrogen phosphate monohydrate, 0.14 g; sodium bicarbonate, 2.2 g; and, glucose 1.0 g. Finally, the volume of the solution is adjusted to 500 ml with water. *Sol.* 2: The following B vitamins are dissolved in 200 ml (final volume) of water: pyridoxine hydrochloride, 25 mg; pyridoxal hydrochloride, 25 mg; i-inositol, 50 mg; p-aminobenzoic acid, 50 mg; and choline chloride, 500 mg. The stock solution consists of a 1:50 dilution of this solution with water. *Sol.* 3: Ten milligrams of d-biotin and 10 mg of folic acid are dissolved in 100 ml of Earle's balanced salt solution(9). The stock solution consists of a 1:100 dilution of this solution with water. *Sol.* 4: Two alcoholic tinctures and one aqueous solution are required to prepare this combined stock solution: cholesterol, 10 mg per ml in 95% ethanol; menadione, 10 mg per ml in 95% ethanol; and, α-tocopherol phosphate, 0.1 mg per ml in water. The following lipid soluble materials are mixed in an aluminum weighing pan: calciferol, 10 mg, dissolved in 1 ml of the tincture of cholesterol; vit. A, 10 mg, dissolved in another 1-ml portion of the tincture of cholesterol; tincture of menadione, 0.1 ml; and, n-butyl parahydroxybenzoate (an antimycotic), reagent grade, 20 mg. Finally, 10 ml of a 5% aqueous solution of Tween 80 are added to a 100-ml volumetric flask together with the alcoholic solutions of vit. A, calciferol, menadione, cholesterol and parahydroxybenzoate. The mixture is then made up to a final volume of 100 ml with water and warmed to dissolve the cholesterol. The stock solution consists of 10 ml of this solution to which is added 1 ml of the α-tocopherol phosphate solution; and, the final volume is adjusted to 100 ml with water. *Sol.* 5: Thirty-six milligrams of ferric nitrate, $Fe(NO_3)_3 \cdot 9\ H_2O$, dissolved in water to make a final volume of 100 ml. One drop of concentrated nitric acid is added to prevent hydrolysis during storage. *Sol.* 6: The following partially purified coenzymes are dissolved in a final volume of 10 ml of water: 70 mg diphosphopyridine nucleotide (DPN), 95% pure; 10 mg triphosphopyridine nucleotide (TPN), 80% pure; 25 mg coenzyme A (CoA) 75% pure; 10 mg cocarboxylase (TPP), 88% pure; 10 mg flavin adenine dinucleotide (FAD), 60% pure; 10 mg uridine triphosphate (UTP), 90% pure. This stock solution is stored in the frozen state and thawed just before use. When large volumes (*e.g.*, 4 liters or more) of medium are prepared, the coenzymes are weighed directly into solution 1. *Sol.* 7: 10 mg of 5-methyldesoxycytidine dis-

‡ 5-methyldesoxycytidine is prepared from wheat germ DNA by the method of Hurst, Marko and Butler(7).

solved in 100 ml of water.

To prepare one liter of solution 858, the various constituents of the final medium (as also the ingredients of solution 1) are mixed in a 5-liter distillation flask with a large central and a small vertical side neck. The large central neck accommodates a bent glass stirring rod attached to a small motor, and the ingredients are added through the side neck. The stock solutions are combined as follows: Solution 1, 500 ml; solutions 2, 3 and 4, 10 ml each; solution 5, 2 ml; solutions 6 and 7, 1 ml each. The final volume is adjusted to 1 liter by the addition of water, and the completed medium is sterilized by passage through UF fritted glass filters (Corning) and stored in the dark at room temperature. Just before use, 1 μg/ml of sodium penicillin G is added.

Results. Nucleic acid constituents. To study the effect of nucleic acids and certain of their derivatives, solution 635 was devised from solution 612 (*i.e.*, solution 199 with added amounts of cysteine, glutathione and ascorbic acid) by omitting the purines, pyrimidines, ATP, adenylic acid, ribose and desoxyribose(1). Solution 635 was capable of yielding 3- to 4-fold increases in the cell population of replicate L strain cultures in one week. When solution 635 was supplemented with highly polymerized calf thymus desoxyribonucleic acid (DNA), or with oligonucleotides resulting from the enzymic hydrolysis of DNA, a 4- to 5-fold increase in the cell population was achieved in one week. The same response was obtained with homologous DNA prepared by the method of Marko and Butler (10) from large populations of L strain cells. In contrast, neither intact ribonucleic acid (RNA) nor its enzymic hydrolysate had any effect on cell multiplication.

Desoxyribonuclease was used to hydrolyze calf thymus and wheat germ DNA to oligonucleotides, and these in turn were hydrolyzed to nucleotides with phosphodiesterase(7). The nucleotides were then separated by ion exchange chromatography, a portion of them were hydrolyzed still further to the corresponding nucleosides, and all 10 hydrolysis products were tested by adding them individually and in groups to solution 635. These assays showed that the 5 desoxynucleosides and the 5 desoxynucleotides could be used interchangeably at an optimal level of 10 μg per ml for each individual substance, with the exception of 5-methyldesoxycytidine, or the corresponding nucleotide 5-methyldesoxycytidylic, both of which were more effective at 0.1 μg per ml. At these levels, the 5 nucleotides and the 5 nucleosides, tested as separate groups, yielded the same growth response as was obtained with intact DNA. Finally, the 5 desoxynucleosides were incorporated in the basal medium.

When solution 635 was supplemented with the individual ribonucleotides and ribonucleosides resulting from the hydrolysis of RNA, adenosine was found to be toxic. Although the other ribonucleotides and ribonucleosides were nontoxic, they had no effect on cell multiplication. When solution 635 was supplemented with the free bases at 10 μg per ml, adenine, guanine, thymine and uracil were toxic, whereas cytosine was nontoxic (1).

Constituents of chondroitin sulfate. Solution No. 199 supported the survival of freshly isolated chick embryo tissues for an average period of 33 days(11,12), though odd cultures that chanced to contain even minute fragments of cartilage continued to live for as long as 170 days(13). In an attempt to discover the particular substance or substances responsible for this prolonged survival, quantitative culture assays were made with various synthetic solutions supplemented with certain commercially available cartilage constituents. None was effective, however, in lengthening the survival of chick embryo tissues or in increasing the rate of proliferation of Earle's L strain cells. Nor were the cultures improved by adding crystalline calcium chondroitin sulfate prepared by the method of Einbinder and Schubert(14) from beef tracheal cartilage. Eventually, a commercial preparation of crude chondroitin sulfuric acid§ (70% pure) was found to be effective (Fig. 3). Because this product had been prepared from cartilage by the classical method of extraction with

§ Supplied by General Biochemicals, Chagrin Falls, Ohio.

strong alkali(15), it seemed possible that the active material might be a hydrolysis product present as an impurity. In any event, chondrosin, chondrosamine, and glucuronic acid were prepared from the crude commercial material by the methods of Levene(16), and of these glucuronic acid gave the same improvement in the cultures as had been obtained with the crude material. Similar results were obtained also with glucuronic acid prepared from 1-naphthol glucuronide and synthetic sodium glucuronate. To establish the *identity of the active material* present in crude chondroitin sulfuric acid, a solution of the latter was placed on an anion exchange resin column (Dowex 1) in the acetate form. Elution with *N* formic acid yielded fractions that gave a positive Dische color test for hexuronic acid (17). When these fractions were combined and concentrated, the final product had growth promoting activity. When the final product was chromatographed on paper together with authentic synthetic sodium glucuronate, the two gave identical R_f values. It seemed probable, therefore, that the activity of the crude commercial preparation of chondroitin sulfuric acid was due to glucuronic acid present as a degradation product. Sodium glucuronate was incorporated in the basal synthetic medium at its most effective level, 0.42 mg%.

Coenzymes and B vitamins. Solution 703 (1) contained 3 highly purified coenzymes, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), and coenzyme A (CoA). Their addition to the medium gave, for the first time, a chemically-defined solution in which animal tissue cells were capable of continuous multiplication and survival. More recently, the effect of these coenzymes has been reinvestigated with the result that they are now used at higher levels; and three additional coenzymes, flavine adenine dinucleotide (FAD), uridine triphosphate (UTP) and cocarboxylase (TPP), have been added to the medium (Table I). Also, 5 of the B vitamins (thiamine, riboflavin, niacin, niacinamide and pantothenate) that are constituents of the coenzymes have been withdrawn from the medium with no adverse effects on the rate of cell multiplication.

Amino acids. It has recently become possible to obtain‖ L-amino acids that are free of D-forms and other isomers. When the natural L-amino acids were incorporated in the medium at one-half the levels previously used for the DL-mixtures, there was a distinct improvement in the appearance of the cultures.

Sol. 858. Replicate cultures of L strain cells in solution 858 (Table I) yield 9- and 10-fold increases in the cell population in 7 days, provided the size of the original inoculum is not much greater than 200,000 or less than 150,000 cells per culture (total medium, 2.5 ml). L strain cells and cells from other sources continue to multiply in solution 858 at a slow rate for considerable periods (Fig. 4-6), though the cells (L strain) that lived for the longest time (141 days) in this solution were lost by contamination.

Supplementation of sol. 858 *with natural body substances.* Solution 858 supplemented with 20% horse serum yields 20- to 30-fold increases in the population of L strain cultures in 7 days, depending upon the size of the original inoculum. In this mixture, L strain cells can be propagated continuously at the same high rate of multiplication and transferred to fresh containers twice a week. The cell yield is not improved by increasing the serum supplement or by adding chick embryo extract to the mixture. In this laboratory, solution 858 supplemented with 20% horse serum is now being used for the continuous propagation of all cell strains, whether from chick, mouse, rat, monkey or man.

Discussion. It is interesting to note that Baker and Ebeling(18), in 1939, included thymus nucleic acid in their semi-synthetic maintenance medium for chick fibroblasts. In the present work, it has been found that L strain cells from the mouse are able to survive and multiply in the presence of highly polymerized DNA, oligonucleotides, nucleotides and nucleosides, but not in the presence of the free bases. It has also been reported by Harris(19) that the free bases are either toxic or inactive when used to supplement a dialyzed

‖ Supplied by California Foundation for Biochemical Research, Los Angeles, Cal.

natural medium for fibroblasts. From the results obtained in supplementation experiments with solution 635, it is apparent that L strain cells can synthesize their own nucleic acids to a limited extent and do not require preformed purines, pyrimidines or pentose sugars. For our basal synthetic medium (Table I), the five desoxyribosides were chosen, instead of the desoxyribotides, so as to make it possible to study the utilization of P^{32} labeled nucleotides by the cells.

It has been noted that the inclusion of crude chondroitin sulfate in the medium had a beneficial effect on cell survival and multiplication, and that this effect was obtained also when the chondroitin sulfate was replaced by glucuronic acid (as sodium glucuronate). Because it is not yet obvious what the metabolic function of glucuronic acid may be, it is difficult to understand how it exerts a beneficial effect on the cells.

Animal feeding experiments have demonstrated repeatedly that the D-isomers of certain amino acids are readily utilized whereas others are not. Thus, D-lysine, D-isoleucine, D-valine and D-threonine are not utilized by the chicken, mouse, rat or man(20). It is impossible, of course, to apply information of this sort directly to the study of explanted cells, but because natural L-form amino acids are now available commercially, it seemed wise to incorporate them in the medium in place of the racemic and diastereoisomeric forms previously used.

Summary. 1. A new synthetic (chemically-defined) medium for animal cells in tissue culture has been devised that is considerably more adequate than solution No. 703, reported earlier. 2. The new medium, solution No. 858, yields 9- and 10-fold increases in the population of replicate cultures prepared from washed suspensions of Earle's L strain cells from the mouse, in 7 days, and supports these and certain other types of cells at a slow rate of multiplication for indefinite periods. 3. Solution 858, the composition of which is given, consists of 62 ingredients that include 3 antibiotics. It differs from solution 703 in that it contains 5 desoxyribonucleosides, sodium glucuronate, and 3 additional coenzymes. Also, 5 B-vitamins present in solution 703 have been omitted, and natural L-amino acids replace the racemic and diastereoisomeric forms previously used. 4. Solution 858 supplemented with 20% horse serum yields 20- to 30-fold increases in the population of L strain cultures in 7 days. In this mixture, L strain cells and cells from other sources can be propagated continuously and transferred to fresh containers twice a week.

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Effect of Bile Acids on Activity of Glucose-6-Phosphatase.* (21720)

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Blood glucose, except for short periods immediately following ingestion of carbohydrate, is maintained by the liver through glycogen breakdown and gluconeogenic processes. In the liver glucose-6-phosphate is the immediate precursor of glucose, and all blood glucose produced by this tissue must arise from the action of glucose-6-phosphatase. In the absence of this enzyme glycogen storage results(1). It has recently been demonstrated that an increase in activity of liver glucose-6-phosphatase per gram of liver is found in fasting and alloxan diabetes(2,3). In interpreting the significance of the increased phosphatase activity found in the alloxan-diabetic liver it is important to know whether the activity is increased by an actual increase in enzyme or whether the increase in activity is the result of the addition of an activator or removal of an inhibitor. Previous studies *in vitro* have not demonstrated the presence of activators or inhibitors that might influence the activity of this enzyme.

Glucose-6-phosphatase is a rather specific organic phosphatase, quite distinct from the so-called alkaline and acid phosphatases, and is found in the microsome subcellular fraction of liver cells(4). Since this enzyme is found in an insoluble phase of high lipoprotein content it occurred to us that surface active agents might influence its activity. Bile acids are predominant among such naturally occurring agents. The ability of bile acids to accelerate the action of lipase has been known for over half a century(5). In addition to lipase the catalytic action of many other enzymes has been found to be influenced by these substances(6). Takata(7) published a series of studies on the effect of bile acids on phosphatases. The hydrolysis of glycerol phosphate and hexosephosphate by alkaline phosphatase of bone, liver and kidney was found to be slightly inhibited (10%) by concentrations of cholic acid from 0.01 to .04%. More recently higher concentrations of bile acids (2%) have been used to solubilize cytochrome preparations and thus allow their study spectrophotometrically(8,9). Succinic oxidase, another mitochondrial enzyme, is inhibited by bile acids and other surface active agents(10,11).

Methods. Glucose-6-phosphatase was assayed by the phosphate release method previously described(2). Livers from normal and alloxan diabetic rats were removed and approximately 0.5 g homogenized in 6 ml of distilled water. Liver microsomes were prepared by differential centrifugation. Whole homogenates were centrifuged for 10 min. at 10,000 x g. to remove the mitochondria and the supernatant solution was centrifuged 90 min. at 100,000 x g. to collect the microsomes. Because of the thermal lability of this enzyme all operations were carried out at 0-5°C. Homogenates and microsomes (0.1 ml) were

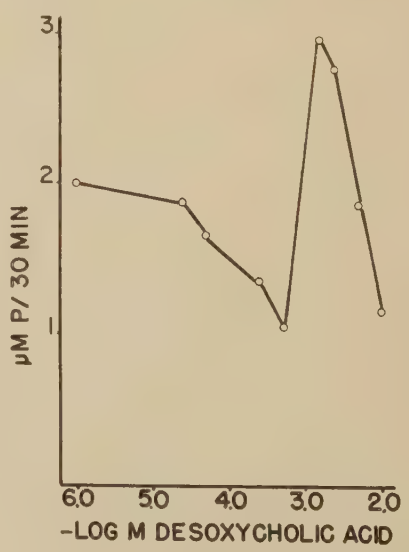

FIG. 1. Effect of desoxycholic acid on rat liver glucose-6-phosphatase. μM of inorganic PO_4 released during 30 min. incubation of 0.1 ml liver homogenate with G-6-P is plotted as a function of – log M concentration of desoxycholic acid in the incubation medium.

* This work was supported in part by the U. S. Atomic Energy Commission.

TABLE I. Effect of Desoxycholic Acid on Glucose-6-Phosphatase Activity. All values are expressed as % of activity found in absence of any added bile acid.

Rats		Mice
Molarity of desoxycholic acid		M desoxycholic acid,
1×10^{-3}	5×10^{-4}	1×10^{-3}
143	70	218
131	57	196
120	71	207
115	76	162
117	75	175
117	68	202
130	83	
123	64	
167	58	
126	77	

incubated for 30 min. at 30°C with 0.2 M glucose-6-phosphate (0.05 ml) in 0.1 M citrate buffer, pH 6.2 (0.2 ml). The effect of bile acids was determined by addition of varying amounts of neutralized solutions of bile acids to the incubation mixture, the total volume of incubation being held at 0.5 ml in all cases and the final pH, 6.7 ± .1. The release of inorganic phosphate was measured by the method of Fiske and Subbarow(12).

Results obtained when desoxycholic acid at various concentrations was incubated with homogenates of normal and alloxan diabetic rat liver are given in Fig. 1. From 10^{-6} to 5×10^{-4} M desoxycholic acid causes a decrease in glucose-6-phosphatase activity. From 5×10^{-4} to 10^{-3} M, desoxycholic acid, the activity of the enzyme increases 3-fold, the total activity at 10^{-3} M desoxycholate being 1.2 to 1.6 times the original activity (Table I). Concentrations of desoxycholic acid greater than 10^{-3} M caused a second decrease in enzyme activity, 0.1 M solutions completely inactivating the enzyme. This type of curve (Fig. 1) was obtained with cholic acid, taurocholic acid and glycocholic acid as well as with desoxycholic acid. When microsome preparations were used, a curve identical with that of homogenates was obtained. The glucose-6-phosphatase activity of mouse liver homogenates responded like the rat liver preparations, except that a greater activation was found at 10^{-3} M, 1.6 to 2.2 times the original activity (Table I).

Since desoxycholic acid present in a concentration of 0.001 M will cause an increase in the activity of glucose-6-phosphatase from normal rat liver comparable to the increase over normal found in the diabetic liver, the influence of this substance was compared in normal and diabetic preparations. Figure 2 demonstrates that glucose-6-phosphatase present in the diabetic liver responds to the addition of bile acid in a manner identical with that of normal liver preparations, the activity of the diabetic preparation always being greater than the normal for every concentration of bile acid tested.

If one plots the rate of glucose-6-phosphate hydrolysis as a function of enzyme concentration both in the presence and absence of added desoxycholic acid (Fig. 3), linear curves are obtained, all passing through the origin. These results indicate that one is justified in using the Michaelis-Menten equation in an attempt to elucidate the mechanism of inhibition and activation(13).

Inhibition and activation analyses were made for concentrations of desoxycholic acid 5×10^{-4} and 10^{-3} M. Lineweaver-Burk plots

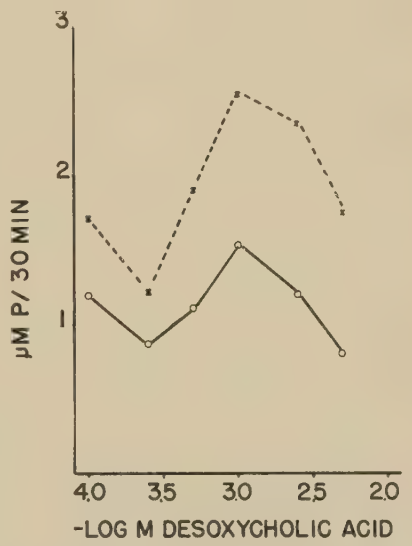

FIG. 2. Comparison of effect of desoxycholic acid on glucose-6-phosphatase activity of liver homogenates from normal (○) and alloxan diabetic (×) rats. μM inorganic PO_4 released during 30 min. incubation of 0.1 ml of liver homogenate with G-6-P at 30°C is plotted against −log M desoxycholic acid concentration.

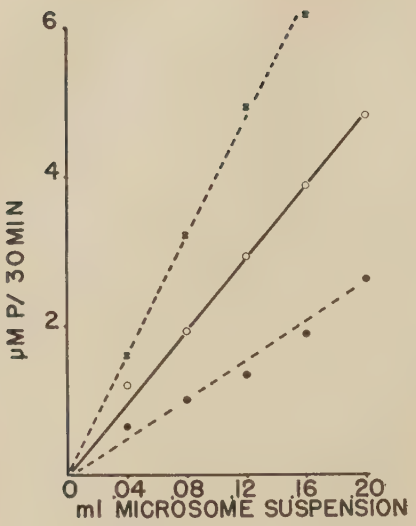

FIG. 3. Effect of desoxycholic acid on rat liver glucose-6-phosphatase with increasing amounts of enzyme. (×---×) Activation by 10^{-3} M desoxycholic acid; (○--○) activity in absence of added bile acid; (●--●) inhibition by 5×10^{-4} M desoxycholic acid. μM inorganic PO_4 released per 30 min. assay is plotted as a function of the concentration of the enzyme (ml of microsome suspension).

for the bile acid at the lower concentration are compatible with the kinetics of a non-competitive inhibitor. However, the same plot, with bile acid present at 10^{-3} M results in a curve of activation in which K_m is not increased. Plotting the rate of reaction as a function of bile acid concentration is grossly non-linear. The change from inhibition to activation produced by different concentrations of bile acids makes it impossible to interpret the entire data in terms of classical Michaelis-Menten theory.

Discussion. It is becoming increasingly evident that many important intracellular enzymes are not in true solution inside the cell, but rather are associated with a predominantly lipid phase. This consideration is even more striking when one considers that the substrates for these enzymes are usually polar substances. Such an arrangement could lead to a very delicate means of regulating enzymatic activity within the cell. In the case of glucose-6-phosphatase the enzymatic activity can be greatly augmented by slight variations in the concentration of bile acids between 5 x 10^{-4} and 10^{-3} M. Since these effects are demonstrable with both free and conjugated bile acids, it is tempting to speculate that the action of these substances is at the lipoprotein-water interface. This would in effect control the substrate concentration in the vicinity of the active surface of the enzyme thus regulating reaction rate. By such a system of activation or inhibition, rates of whole systems of enzymes could be regulated by one substance. Many naturally occurring materials, notably the steroid hormones(14) might conceivably act by such a mechanism.

It is difficult to assign any mechanism for the action of the bile acids on glucose-6-phosphatase from these studies. However, certain facts stand out. These effects are not specific for any one bile acid or any one conjugated form. They are consistently reproducible and apparently do not greatly influence the enzyme substrate dissociation constant. High concentrations of the bile acid, sufficient to solubilize the microsomes result in loss of all enzymatic activity. The recent studies by Beaufay and de Duve(15) would also indicate that conditions which lead to dissolution of the microsomes result in inactivation of glucose-6-phosphatase. Thus, one is not dealing with an activation due solely to solution of the protein, but probably with a closely related phenomenon. It is probable that two processes are going on simultaneously; a direct combination of enzyme and bile acid resulting in inactivation and some other effect resulting in activation, the observed response being the sum of these two independent effects.

Since glucose-6-phosphatase responds to the activation effects of the bile acids both at normal activities and the increased activities of the diabetic, it is unlikely that the increased activity of this enzyme in diabetic liver preparations can be the result of such an activation. This conclusion is further strengthened by the observation that 12-14 hours are required to produce *in vivo* changes in the activity of glucose-6-phosphatase.

Summary. (1) Bile acids have been found to influence, *in vitro,* the activity of rat liver glucose-6-phosphatase, concentrations of 5 x 10^{-4} M inactivating and 1 x 10^{-3} M activating the enzyme. (2) Glucose-6-phosphatase

from both normal and diabetic rats responds similarly. The increased glucose-6-phosphatase activity found in liver of alloxan diabetic rats therefore does not appear to be due to any such activation mechanism, but rather due to an actual increase in the amount of enzyme present.

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Plasma Thromboplastin Component (PTC) Deficiency Produced by Prolonged Administration of Prothrombopenic Anticoagulants.* (21721)

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(Introduced by Joseph M. Hayman, Jr.)

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In an investigation of the effect of phenindione (PID) on the coagulation mechanism (1), it was discovered that patients who had received this anticoagulant for more than about 30 days developed a distinctly prolonged glass clotting time. Since there was no detectable change in the degree of deficiency of prothrombin or of proconvertin (Factor VII, Co-thromboplastin, SPCA, Stable Factor) at this time, another factor was suspected to be deficient, and these suspicions prompted investigation of the thromboplastin complex. Previous unpublished experiments of ours had excluded antihemophilic factor deficiency and investigation for the presence of Plasma Thromboplastin Component (Christmas factor, Antihemophilic factor B) seemed indicated. The 3 reports on this factor(2-4) as a member of the thromboplastin complex have clearly shown its identity as a separate component; and have shown that a deficiency is associated with a hemorrhagic diathesis with a prolonged glass clotting time and a clinical picture indistinguishable from classical hemophilia. Methods of assaying for the presence of PTC have been the prothrombin consumption with and without barium sulfate adsorbed plasma and serum added as corrective factors (5), prothrombin consumption with plasma of known patients with deficiencies added as corrective factors, glass clotting time, recalcification time, and the "thromboplastin generation test" as described by Biggs and Douglas(6). On the basis of the reports in the literature, the prothrombin consumption and the thromboplastin generation test were chosen as being the most accurate available.

Methods. All blood was collected carefully using only a clean venipuncture with Arquad coated needles and silicone coated syringes. One part 0.1 M sodium oxalate to 9 parts whole blood was used as anticoagulant for collection of plasma. Blood for serum was allowed to clot, incubated one hour at 37°C,

* Supported by grants from National Heart Institute and the Massachusetts Heart Assn.

then oxalated, and finally incubated another period of one-half hour at 37°C. Plasma and serum thus collected were stored in a frozen state at −20°C until used in the prothrombin consumption test. The prothrombin consumption test was performed according to the method of Stefanini(7), the times being converted to per cent by barium sulfate dilution curve. Subjects were surveyed for the presence of PTC by observing the corrective effect of 0.1 ml of their plasma on the prothrombin consumption of 1.0 ml of whole blood from a subject with PTC deficiency (J.G.)†, and compared with the corrective effect of 0.1 ml of saline, normal plasma, barium sulfate adsorbed plasma, and serum. The thromboplastin generation test was done according to the method of Biggs and Douglas(6) with the following modifications: 0.1 M sodium oxalate was used as the anticoagulant for collection of plasma instead of sodium citrate; barium sulfate, 40 mg/ml was used for adsorbing the plasma instead of alumina gel, and the sera were oxalated after clotting in the manner described above. Since the serum of patients on PID is deficient in proconvertin, this factor was supplied without PTC by using a mixture of 75% whole blood from a patient with PTC deficiency (R.G.)† and 25% whole blood from the patient to be tested. These bloods were collected in silicone tubes in an ice water bath, transferred with silicone coated pipettes in required amounts to glass tubes, mixed, transferred to a water bath at 37°C, allowed to clot, and the serum then treated as in the usual thromboplastin generation test. All patients on phenindione had a prothrombin time between 22 and 32 seconds at the time of using their plasmas in the tests. The patients with PTC deficiency were identified by the failure of correction by $BaSO_4$ plasma and correction by aged normal serum and by plasma of a patient with classical hemophilia of the prothrombin consumption test(2,5). In addition, these patients showed an abnormal thromboplastin generation test when their serum and a normal result when their $BaSO_4$ plasma was used in the thromboplastin generation test. Both had a normal proconvertin content of the plasma and thus according to Biggs and Douglas(6) this identifies PTC deficiency.

† Two members of the same family in which the defect is transmitted by sex linked recessive trait.

TABLE I. Serum Prothrombin of Patient (J.G.) with PTC Deficiency when Various Corrective Factors Are Added.

Corrective factor			Serum prothrombin, %
None			52*
Saline			76
Plasma of patient with classical hemophilia (Hemophilia A)			20
Normal plasma			18
$BaSO_4$ fresh normal plasma			74
Aged normal serum			10
Plasma of patient with PTC deficiency (R.G.)			77
Plasma Patient 1 on phenindione for		mos.	62
2			60
3			12
4			18
5			13
6			16
7			52
8			56
9			12
10		4 days	9
11		9	11
12		19	19
13		27	62
14	dicoumarol	mos.	56

* Underlined figures signify failure of correction.

Results. Prothrombin consumption. The abnormal prothrombin consumption of the patient with PTC deficiency was corrected by normal serum and by most of the plasmas of patients on phenindione. The plasmas of 5 of 10 patients on phenindione for 27 days or more and of one patient on dicoumarol for months, however, failed to correct the defect in prothrombin consumption. It is suggested that the factor which was missing in the blood of the patient with PTC deficiency was also missing in these 5 patients (Table I).

Thromboplastin generation test. Three individuals from the group were selected (patients 2, 4, 7) of whom 2 did not correct and one did correct the abnormal prothrombin consumption of the patient with PTC deficiency. The thromboplastin generation test reflected the same degree of correction as the prothrombin consumption test in these cases as may be noted in Fig. 1. Of the 3 on short term phenindione, one who had been on phenindione 9

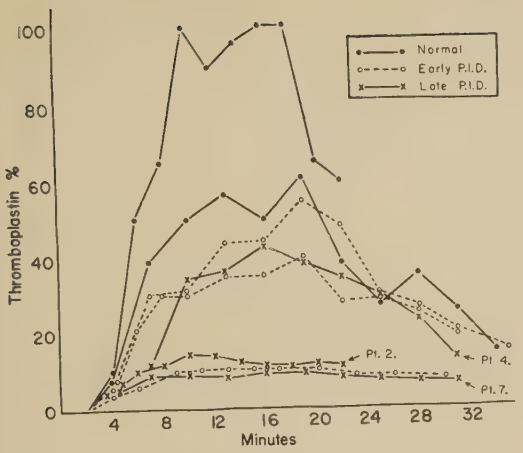

FIG. 1. Thromboplastin generation test using a mixture consisting of 3 parts whole silicone blood from a patient with PTC deficiency and 1 part whole silicone blood from subject to be assayed. Bloods after mixing were incubated at 37°C in glass tubes, and after clotting the sera were then used in thromboplastin generation test with fresh normal barium sulfated plasma and normal platelets. "Early P.I.D." indicates patients receiving phenindione for 3-9 days. "Late P.I.D." indicates patients receiving phenindione for months on long term anticoagulant program.

days showed no correction which was unexpected while the other two did. It is clear that the defective thromboplastin generation in the 3 subjects must have been due to a deficiency of other than proconvertin because the other 3 subjects who were equally deficient in proconvertin showed normal thromboplastin generation. One patient (Case 2) stopped phenindione and was restarted. One week later, when the prothrombin time was 24 seconds, the thromboplastin generation was normal.

Discussion. Phenindione as well as dicoumarol is known to inhibit elaboration of prothrombin and proconvertin. The evidence here would indicate that it also inhibits the production of PTC. That prothrombin and proconvertin have a short survival in the host is well known by transfusion experiments. Therefore, if the manufacture of these factors is stopped, a deficiency will develop rapidly. On the other hand, there is evidence that the beneficial effect of a transfusion may be seen for many days in PTC deficiency(2). Consequently, if the production of PTC by the liver were stopped, a deficiency of a measurable degree would not develop for a long time. Of further comparative interest is that prothrombin, proconvertin, and PTC are all adsorbable on $BaSO_4$.

Summary. Evidence is presented showing that a deficiency of PTC (Hemophilic factor B, Christmas factor) may develop after prolonged administration of phenindione and also probably dicoumarol.

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Interaction of Vasopressin With Adrenocorticotrophic Hormone, Cortisone and Somatotrophic Hormone; Possible Relation to Eclamptic Convulsions. (21722)

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The convulsive seizures of the eclamptic state have been associated with water retention as a result of the action of an anti-diuretic factor(1). Although the precise nature of this factor is unknown, it has been suggested recently that it may be vasopressin(2). Experimentally, administration of vasopressin in normal rats has been shown to increase the excitability of the central nervous system(3) but the dose of vasopressin used was large. This would seem to cast doubt on the part played by vasopressin in the onset of eclamptic convulsions. There is however, little doubt that eclamptic patients are more sensitive to the vascular and renal effects of vasopressin than are normal pregnant women. This has been effectively demonstrated by Dieckmann and Michel(4). It has been suggested that this sensitization to vasopressin may be due to the concomitant presence of certain other hormones, namely, those of the anterior pituitary and adrenal cortex(5).

It would seem possible from the foregoing, that the convulsions of eclampsia might be due to the action of vasopressin on a central nervous system made sensitive to stimulation by the presence of other hormones. This possibility of sensitization has been investigated in rats and this is a report of the results.

Materials and methods. Sprague-Dawley male rats, weighing approximately 120-200 g were used throughout. The method for determining reaction of the central nervous system to various hormones is based on the measurement of the electroshock threshold (EST) as described by Swinyard(6). This method was modified by applying electroshocks which had the following characteristics: 100 rectangular pulses/sec., 1 msec. pulse duration; for 0.3 seconds. The threshold is the smallest amperage which produces a detectable clonic seizure. The following procedure was carried out in each experiment: the EST measurements were made once a day for 5 or more days until the thresholds were stabilized and did not vary more than ± 0.5 ma. per day. The injection of the various hormones were started following stabilization of the EST and the thresholds were determined once daily, 4 hours after injection for a period of 5 days for each treatment. Between treatments the animals were allowed to recover, until the time when the EST had returned to the pre-treatment level. The hormones were dissolved in 0.9% NaCl with the exception of cortisone acetate which was obtained already dissolved in a special vehicle.* All hormones were administered subcutaneously in the following doses: vasopressin, 0.1 unit/100 g per day,† adrenocorticotropic hormone (ACTH, Armour), 1 unit/total dose per day, cortisone acetate (Schering), 0.8 mg/100 g per day and somatotropic hormone (STH, F. W. Horner, Ltd.), 1 mg/total dose per day. These doses were chosen because they are within the dosage range which has been shown to evoke a response in the EST in rats(3,7). Three experiments were conducted. The effect of ACTH alone or in combination with vasopressin was studied in Exp. I, the effect of STH alone or in combination with vasopressin was studied in Exp. II and the effect of cortisone acetate alone or in combination with vasopressin was studied in Exp. III.

Results. The results of these 3 experiments are summarized in Table I. As shown in a previous report(3) vasopressin again lowered the EST in all the experimental groups. The response to vasopressin appears to be a rather consistent one and was roughly the same in each experiment. Administration of ACTH or cortisone acetate together with vasopressin produced a significant lowering of the EST which was greater than the effect of vasopres-

* The vehicle for dissolving cortisone acetate (Schering) contains: potassium biphosphate, exsiccated sodium phosphate, polysorbate 80, sorbitan monolaurate, thimerosal and distilled water.

† I am indebted to Dr. D. A. McGinty of the Parke, Davis Co. for supplying me with vasopressin.

TABLE I. Effect on EST of ACTH, STH and Cortisone, Alone or in Combination with Vasopressin, 20 Rats in Each Experiment.

Exp.	Treatment	EST,* ma	% change	P†
1	Before treatment	14.7 ± 1.7		
	Vasopressin	11.1 ± 1.7	−24.5	<.01
	" + ACTH‡	8.0 ± 2.3	−45.6	<.01
	ACTH	12.5 ± 3.0	−15.0	>.05
2	Before treatment	24.9 ± 1.0		
	Vasopressin	17.0 ± 3.5	−31.9	<.01
	" + STH	31.6 ± 4.7	+26.9	<.01
	STH	31.7 ± 5.8	+27.3	<.01
3	Before treatment	17.5 ± 0.87		
	Vasopressin	11.9 ± 2.66	−32.0	<.01
	Vasopressin + cortisone‡	9.5 ± 0.81	−46.7	<.01
	Cortisone	12.9 ± 0.59	−26.3	<.01

* Mean and stand. dev.

† P = Probability from Fischer's table of "t."

‡ Between treatment with vasopressin and vasopressin plus ACTH P = <0.01. Between treatment with vasopressin and vasopressin plus STH P = <0.01. Between treatment with vasopressin and vasopressin plus cortisone P = <0.01. Between treatment with cortisone and vasopressin plus cortisone P = <0.01.

sin alone or of either hormone alone.

The observation that cortisone lowered seizure thresholds when given together with vasopressin may indicate a synergistic effect of 2 similar actions, in view of other reports of the EST lowering effect of cortisone alone (7,8). It seems possible then that the effect of ACTH upon vasopressin-induced lowering of the EST was due to the release of adrenal cortical steroids, among them cortisone, from the adrenal cortex through the stimulus of ACTH. One cannot, however, exclude the possibility that ACTH evoked its effect on EST by a direct action on the central nervous system or that the effects of cortisone or ACTH on the response to vasopressin did not result from a simple synergism of 2 different actions. The interaction of ACTH and cortisone with vasopressin and their effect on EST may have pertinence with regard to the role of vasopressin in inducing eclamptic convulsions for Tobian(9) has shown that adrenal cortical activity is increased in pre-eclamptic patients.

When STH and vasopressin were given together, the EST was significantly elevated and the effect of vasopressin was completely prevented by STH. Selye(10) and Li *et al.*(11) have found that some of the actions of STH resemble those of desoxycorticosterone (DC). DC has previously been shown to be capable of antagonizing the effects of vasopressin on the central nervous system and elevating the normal EST when given alone(7,12). It seems possible in theory that DC may be the steroid, secreted by the adrenal cortex under the influence of the tropic hormone STH, which tends to prevent the hyperexcitability of the central nervous system induced by vasopressin.

Summary. The effect on EST of the hormones ACTH, cortisone acetate and STH alone and in combination with vasopressin has been investigated. ACTH and cortisone acetate in combination with vasopressin each produced a greater fall in EST than was produced by vasopressin alone. In contrast, STH in combination with vasopressin prevented the fall induced by vasopressin alone and produced its usual elevation in the EST. These observations indicate that the tropic hormones ACTH and STH may modify the response of the central nervous system to vasopressin, possibly through their influence on the adrenal cortex. This interaction of hormonal effects may be involved in the convulsions of eclampsia.

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Local Increase of Poliomyelitis Virus in Healing Wound Tissue of Cynomolgus Monkeys.*† (21723)

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The concept that extraneural multiplication of poliomyelitis virus is important in the pathogenesis of poliomyelitis has gained increasing support during the past 5 years since this virus has been shown to multiply in a variety of nonneural tissues in tissue culture. Attempts to demonstrate *in vivo* multiplication in extraneural tissues have met with failure when essentially normal animals were used(1,2). Cortisone treated hamsters and monkeys have been shown to be susceptible to the multiplication of poliomyelitis virus in certain extraneural tissues, notably brown fat, according to Shwartzman and Aronson(3,4). The present paper concerns experiments in which poliomyelitis virus was noted to increase at the site of inoculation into healing skin wounds on the backs of cynomolgus monkeys.

Materials. Two male cynomolgus monkeys weighing 9¼ and 9¾ lb. were used in these experiments. Both animals had been housed in our laboratory for more than 2 years. Neutralization tests with serum collected shortly before the present experiments showed no antibodies to any of the 3 types of poliomyelitis virus. Type 3 poliomyelitis virus of the Saukett strain was obtained from the Connaught Laboratories as frozen fluid from monkey kidney tissue. This was the standard strain of tissue cultured virus used in the serological tests in the vaccine evaluation program sponsored by the National Foundation for Infantile Paralysis. Hanks' solution was used as diluent for the virus. The ID_{50} calculated by the method of Reed and Muench was $10^{-4.25}$. This was based on a titration carried out immediately after inoculation of the monkeys. Serial 10-fold dilutions of virus were prepared, and each dilution was inoculated into 5 tissue cultures. *Assay of virus* in the inoculum and in the exudate from inoculated sites on monkeys was carried out by titration in tissue cultures of monkey kidney. Minced cortical tissue, suspended in phosphate buffer with trypsin, was agitated in a Waring blendor until a cell suspension was obtained. The procedure was essentially that of Youngner(5). After several washings the cells were suspended in medium, inoculated into test tubes and incubated for periods of 5 to 7 days at 37°. In some instances they were stored for a period of a few days at 20°C. In all instances titrations were carried out by diluting the virus in either Hanks' solution or tissue culture medium and inoculating tubes with 0.1 ml of each dilution. One-half ml of medium 199 with 2% horse serum was used with all cultures during the period prior to inoculation. At the time of inoculation medium was poured off and 0.4 ml of fresh medium 199 was added. Penicillin, streptomycin and phenol red were included.‡ Results are expressed in terms of infectivity of the inoculum rather than of the calculated dilution in the medium after inoculation. Control tissue cultures, usually numbering from 4 to 6, were used with all titrations. The criterion for infection was degeneration of cells with the appearance characteristic of poliomyelitic infection, progressing to nearly complete destruction of the cell population by 7 days.

Procedures. Preparation of skin wounds. The monkeys were anesthetized with nembutal, the hair of the back removed by shaving, and the skin washed with soap and water. The sites of all future skin wounds were then marked with India ink on the back. One square centimeter of skin was removed from

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‡ Medium 199 was obtained from Microbiological Associates, Bethesda, Md. As received, it contained no $NaHCO_3$. The final concentration of $NaHCO_3$ was 0.084% in the medium used prior to inoculation and 0.125% in the medium used after inoculation.

each of 3 areas. The lesions were 1.5 cm apart. Excision of 3 pieces of skin was carried out on each of 3 successive days in the case of 1 monkey and 4 successive days in the case of the other monkey. The lesions were arranged in 2 vertical rows equidistant from the midline. Two per cent procaine hydrochloride, with epinephrine 1 to 50,000, was employed as a local anesthetic for the excision of skin after the first day. Six hundred thousand units of procaine penicillin and 500 mg of streptomycin were injected intramuscularly each day that the monkeys were handled for the excision of skin. These antibiotics were also administered on the day of inoculation and at 48-hour intervals during the period when exudate was harvested for virus assay. With few exceptions there appeared to be no bacterial infection of these lesions throughout the experiment. Inoculation of virus was carried out when the lesions of monkey X446 were 3 to 6 days old and those of monkey X460 were 3 to 5 days old. Two methods were employed. In view of the results obtained, it does not appear that these two methods differed in their efficacy. In one method virus was injected into the tissue immediately under the crust. In the other method, the crust was removed and virus was administered to the tissue at the base of the lesion by injection, by massaging with a cotton swab soaked with virus suspension, and by multiple puncture. In all cases a 10^{-1} concentration of virus suspension was employed. (The material as received was considered 10^{-0}.)

Two lesions in each group of 3 were inoculated with removal of the crust; the third was inoculated with the crust intact. The volume of the inoculum in lesions with the intact crust was .03 to .05 ml. The total virus administered to lesions in which the crust was removed was approximately 0.1 ml. Exudate was harvested for virus assay daily during the first 5 days after inoculation and at 7 and 10 days. The crust, which had formed since the previous harvesting, was removed and placed in a tube with 1.5 ml of sterile water. A cotton swab was then used to take up all fluid exudate possible from the base of the lesion. This swab was rinsed in 1.5 ml of sterile water in a second tube. The swab was used to gently massage the base of the lesion and rinsed a second time in the same tube of water. The swab was discarded. Tubes with crusts and with the rinsings of the exudate were stoppered and frozen at –40°C for future assay. One week after inoculation a number of the lesions showed a marked healing and decrease in size. At 10 days 3 of the lesions were completely closed, and 3 more were healed to the point where no scab was present. All showed marked reduction in size.

Results. Preliminary titration of pooled samples indicated that an increase of virus had occurred during the course of the experiment. Therefore, further virus assays were carried out using material from selected individual lesions.

In Table I the results of assays of virus from all lesions are presented. Amounts of exudate were not determined in this experiment, but in a subsequent experiment with rhesus monkeys this was done at 1 to 7 days after inoculation of lesions 3 and 6 days old. From 5 to 30 mg of exudate were obtained. There was less with increasing age of lesions. Most lesions yielded 10 to 15 mg. Therefore, the original harvest of exudate into 1.5 ml of water is estimated to represent a 10^{-2} dilution of exudate in the present experiment.

Data for lesions J and K of monkey X446 and lesions G and I of monkey X460 indicate clearly that the amount of virus in the exudate was less at 1 day than at 3 and 5 days. There appears to have been an increase of approximately 1000-fold in the amount of virus during this time interval. It further appears that in lesion L of monkey X446 there was very little virus present on the first day, and that a subsequent increase did not occur.

Lesions that were 5 or 6 days old when inoculated contained more virus at 1 day than lesions that were 3 days old at the time of inoculation. Maximal amounts of virus at 3 and 5 days were approximately the same in lesions 3 and 5 or 6 days old at the time of inoculation. Seven of the 8 lesions tested at 7 days were positive for virus. Unexplained failure to infect tissue cultures with lower dilutions of exudate occurred in the case of lesions C and H of monkey X460. The con-

TABLE I. Tests for Poliomyelitis Virus in Exudate of Skin Lesions.

Monkey X446

Age of lesion when inoculated	3 days										4 days			5 days			6 days														
Lesion	J			K				L			G	H	I	D	E	F	A					B				C					
Time of harvest in days	1	3	5	1	3	5	7	1	3	5	5	5	5	5	5	5	1	3	5	7	10	1	3	5	7	1	3	5	7	10	
Dilution of exudate																															
10^{-7}			0/2														0/2		0/2												
10^{-6}		0/2	1/2		1/2	0/2	0/2										”	0/2	”								0/2	”	0/2		
10^{-5}		”	2/2		”	1/2	”		0/2	0/2		1/2	1/2	0/2	0/2	0/2	1/2	”	2/2	0/2				0/2		0/2	”	1/2			
10^{-4}	0/2	2/2	”	0/2	2/2	2/2	2/2	0/2	”	”	0/2	2/2	2/2	2/2	”	”	2/2	2/2	”	2/2	0/2		0/2	”	0/2	”	1/2	”	0/2		
10^{-3}	”			”	”	”	”	”			”	”	”	”	1/2	1/2	”			”	”	0/2	2/2	1/2	”	2/2	2/2	2/2	”	0/2	
10^{-2}	2/2			”	”	”	”	1/2			2/2	”	”		2/2	2/2				”	”	2/2	”	2/2	1/2	”			2/2	”		

Monkey X460

Age of lesion when inoculated	3 days										4 days			5 days																	
Lesion	G			H				I			D	E	F	A				B				C									
Time of harvest in days	1	3	5	1	3	5	7	1	3	5	5	5	5	1	3	5	7	1	3	5	7	1	3	5	7	10					
Dilution of exudate																															
10^{-7}		0/2	0/2						0/2					0/2																	
10^{-6}		”	1/2				C		”	0/2				”	0/2	0/2					0/2				0/2	1/2					
10^{-5}		2/2	”		0/2		C		2/2	”			0/2	1/2	”	”				1/2	1/2	0/2	0/2	”	”						
10^{-4}	0/2	”	”		”		C1/2	0/2	”	1/2			1/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	”	”	”	1/2	2/2	1/2	0/2				
10^{-3}	”			1/2	1/2	3/4	1/3	”					2/2	”			”	”	”	”	”	2/2	2/2			0/2	”				
10^{-2}	2/2			2/2	2/2	4/4	0/4	1/2			2/2	2/2	”	”			C	”	”	”	”	”				C	0/1				

Two tissue cultures of rhesus kidney cells were inoculated with each specimen. Degeneration of cells within 7 days with characteristic morphology was accepted as evidence of infection. 2/2 = 2 tissue cultures inoculated and both were infected. C = contamination with bacteria.

tinued presence of virus in a lesion did not stop or noticeably retard the healing process.

It appears possible that the relatively large amounts of virus present at 24 hours in the lesions 5 and 6 days old may have represented early multiplication of virus.

Discussion. Poliomyelitis virus appears to multiply much more readily and abundantly in roller tube cultures of rhesus monkey testis than in suspended cell cultures of the same tissue(6). The multiplication of virus occurs in fibroblast-like cells that proliferate in the roller tube cultures(7). In suspended cell cultures of the kind that have been employed with rhesus testicular tissue there is little fibroblastic proliferation. It was reasoned that if one were to create a lesion in the monkey skin and inoculate virus after a lapse of time sufficient for fibroblasts to multiply, a susceptible tissue might then be available for multiplication of poliomyelitis virus.

The results that have been obtained demonstrate that the amount of virus at the site of inoculation increased in amount as might be expected in tissue cultures. Whether this increase was due to the mechanism hypothesized is not demonstrated. It is possible that virus traveled the short distance to the central nervous system from the inoculated lesions, multiplied there, and returned by way of nerve fibers to the site of inoculation. In our judgment this does not appear to be the most probable mechanism, but it is not ruled out by the evidence presented here.

Definite evidence of increase of virus is presented in the case of 4 of the 3-day lesions. It appears that virus may have multiplied within the first 24 hours in lesions that were 5 or 6 days old at the time of inoculation. Further experiments involving assay of virus at intervals during the first 24 hours are necessary to settle this point.

Conclusions. 1. Pieces of skin 1 cm square were removed from the backs of 2 mature male cynomolgus monkeys. When the sites of excision were 3 to 6 days old, poliomyelitis virus of the Saukett strain was inoculated into the lesions. In the 6 lesions, 3 days old at the time of inoculation, the concentration of virus was minimal at 1 day. Subsequent increase of virus occurred in the case of 4 of these lesions. 2. The amount of virus in the exudate from lesions 5 and 6 days old at the time of inoculation was close to a maximal level at 1 day. At 5 days virus was present in approximately maximal concentration in 11 of the 12 lesions. Virus was present in 7 of the 8 lesions tested for virus one week after inoculation. Extensive healing of wounds had occurred by this time.

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Coagulase in Reversing Antibacterial Activity of Normal Human Serum on *Micrococcus pyogenes.** (21724)

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Although coagulase activity has been known since the early work of Loeb(1), and used as a criterion of pathogenicity for the *Micrococci* since the work of Much(2), its mechanism of action in the pathogenesis of staphylococcal infections is still enigmatic. The work of Smith and Hale(3-5) has increased our knowledge of the mode of action of coagulase *in vitro,* and has given some indication of its action *in vivo.* These workers have presented evidence that coagulase interferes with phagocytosis, although van Heyningen(6) believes that this activity could have been of a purely mechanical nature since both the cocci and the leukocytes tended to aggregate in clumps in the fibrin. Raffel(7) states that even though coagulase "provides a simple test for the probable pathogenic significance of a staphylococcus isolated from a lesion—there is as yet no reasonable suggestion as to its possible significance with respect to pathogenicity. It is possible that coagulase may exert some yet unknown influence upon tissues or fluids aside from its clotting activity."

Evidence presented here indicates that coagulase may function to inhibit the antibacterial activity of normal serum upon certain strains of *M. pyogenes* susceptible to this serum factor.

Materials and methods. The organisms used in this study were obtained from the University Hospital and unless otherwise indicated were recent isolates from human sources. They were grown in pure culture in brain heart infusion (BHI) broth, and stored at –50°C until used. *The serum* was obtained from human volunteers and was separated from the cellular elements of the blood as soon as the blood had clotted. It was either used immediately in the antibacterial test or stored at –50°C until used. Not more than one hour elapsed between the time that blood was drawn and the time it was used or stored in the frozen state. In all tests at least 3 individual serum samples were pooled. *The antibacterial test* was carried out by inoculating 0.1 ml of a 10^{-6} dilution of an 18 hour BHI broth culture of the organisms into 1.0 ml of undiluted serum. The test was performed in serological tubes 10x75 mm, which were kept capped with rubber stoppers between samplings. This procedure was found to be adequate in controlling the alkaline shift of serum due to the loss of carbon dioxide. Using this technic the pH was held within a physiological range over 24 hours without the introduction of additional buffers. Duplicate 0.1 ml aliquots were removed immediately after inoculation and at the designated intervals of time and plated in BHI agar either directly or after appropriate dilution in saline to obtain countable plates. Pour plate technic was used and colony counts were made after incubation at 37°C for 24-48 hours. The experimental tubes were incubated in a water bath during the test. *Coagulase* was prepared by ammonium sulfate precipitation of the culture fluid after growth of a coagulase positive strain for 48 hours at 37°C. The cultures were grown in 200 ml amounts in BHI broth with constant agitation on a shaker. The supernatant fluid after sedimentation of the cells by centrifugation was dialyzed against saturated ammonium sulfate for 48 hours at 5°C. Solid ammonium sulfate was present at all times in the fluid surrounding the dialysis tubing. The resulting precipitate was separated by centrifugation in a Sorvall angle head centrifuge at 10,000 rpm, dialyzed free of salt in running tap water, and dried by lyophilization. Coagulase activity of the culture supernatants and the

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partially purified products was determined by mixing 0.5 ml of the material to be tested with an equal volume of human plasma† diluted 1:5. This mixture was incubated at 37°C for 4 hours followed by overnight incubation at room temperature. The product to be titrated was prepared in a concentration of 1.0 mg per ml and serially diluted. The titer was taken as the highest dilution showing any visible clot after the overnight incubation. *Serum* was treated with the purified coagulase preparations by dissolving the material directly in the serum in varying amounts. Since aseptic preparation of the coagulase would not have been feasible, the serum samples so treated were filtered through a Berkefeld N filter before being used in the antibacterial tests. Control experiments showed that such treatment of the serum did not affect its antibacterial activity against the susceptible strains of *M. pyogenes*.

Results. Conflicting evidence concerning the bactericidal effect of human serum upon various strains of *Micrococcus* has been presented in past years. Thus Flexner(8), Idelsson(9), Much(10), and Selter(11) have reported definite bactericidal activity of serum on these organisms, while White(12), Wright and Windsor(13), Seiffert(14), Mackie and Finkelstein(15), Wulff(16), and Tejler(17) were unable to demonstrate bactericidal action with human serum. This seeming discrepancy in results can be explained when it is pointed out that in each case where a bactericidal effect was not demonstrated the authors had considered only organisms which had been isolated from pathological processes and presumably were in a reasonably virulent state. In instances where saprophytic members of the group were included in the studies distinct antibacterial activity could be demonstrated.

By carrying out the antibacterial test in such a way that the actual growth of small inocula of the organisms in the serum could be followed, instead of mere percentage survival of relatively large inocula after short exposures to serum, as is done in some cases, a definite bacteriostatic activity could be demonstrated with the coagulase negative, avirulent strains. Those strains which were coagulase positive, virulent strains, were resistant to the antibacterial activity of normal human serum and grew luxuriantly under the conditions of the experiment. The results of a survey of 35 strains of *M. pyogenes* as to their biochemical activities and their ability to grow in human serum are presented in Table I. The close correlation between coagulase positivity and growth in serum is clearly evident. Other metabolic products of the organisms appeared to have no direct relation to the *in vitro* growth of the serum resistant strains in

† Obtained from recently outdated transfusion blood from the University Hospital blood bank.

TABLE I. Survey of 35 Strains of *Micrococcus pyogenes* with Respect to Physiological Properties and Growth in Normal Human Serum.

Coagulase production	Pigment	Mannitol fermentation	Toxins		Growth in serum*
			Alpha	Beta	
+	+	—	—	—	4
+	+	+	+	+	4
+	+	+	—	—	4
+	+	+	+	—	4
+	+	+	—	—	4
+	+	+	+	—	4
+	+	+	+	—	4
+	+	+	+	—	4
+	+	+	+	+	4
+	+	+	+	—	4
+	+	+	+	—	4
+	+	+	+	—	4
+	+	+	+	—	4
+ —	+	+	—	—	0
+ —	+	—	—	—	1
—	—	—	—	—	0
—	—	—	—	—	0
—	—	—	—	—	0
—	—	—	—	—	0
—	—	—	—	—	0
—	—	—	—	—	0
—	—	—	—	—	0
—	—	—	—	—	1
—	—	—	—	—	0
+ —	+	+	—	—	0
+ —	+	+	—	+	1
+	+	+	+	+	2
+	—	+	+	+	0
+	+	+	+	+	4
+	+	+	+	—	4
+	+	+	+	—	4
+	+	+	+	—	4
+	+	+	+	—	4
—	—	—	—	—	1
—	—	—	—	—	0

* 4—indicates confluent growth on 24 hr plating of 0.1 ml undiluted serum sample. 0—indicates fewer colonies on 24 hr plating than at 0 time plating.

TABLE II. Effect of Ammonium Sulfate Precipitated Culture Filtrate Material from Serum Susceptible and Serum Resistant Strains of *Micrococcus pyogenes* upon Growth of Susceptible Strains in Human Serum.

Strain No.	Serum treated with resistant strain filtrate material* 0 hr	24 hr	Serum treated with susceptible strain filtrate material 0 hr	24 hr
1	1.3×10^2†	5.0×10^7	1.5×10^2	1.2×10^2
2	5.0×10^1	8.0×10^6	5.0×10^1	4.0×10^1
3	3.0×10^2	1.8×10^7	5.0×10^1	2.0×10^1

* 1 mg ammonium sulfate saturated culture filtrate precipitate/1 ml of serum.
† Plate counts per 1 ml.

normal human serum. With this observation in mind it was reasoned that if coagulase was functioning to protect the resistant strains against the antibacterial activity of serum, by addition of cell free exogenous coagulase to serum, coagulase negative serum susceptible strains of *M. pyogenes* might be induced to grow in otherwise inhibitory serum. Preliminary experiments with crude culture filtrates from coagulase positive serum resistant strains indicated that this might be the case.

The coagulase preparation obtained by ammonium sulfate saturation of the culture filtrate had a titer such that 10 μg of the dry material produced a solid clot in human plasma diluted 1:5 in less than one hour at 37°C. The alpha toxin titer was such that 320 μg of the dry material produced complete hemolysis of 1.0 ml of 0.5% rabbit red blood cells. No beta toxin could be demonstrated in this preparation. The alpha toxin titer could be completely destroyed by heating at 65°C for 30 minutes without affecting the coagulase titer. The serum was treated with the equivalent of 1.0 mg of the dried coagulase preparation per ml of serum. The identical ammonium sulfate fractionation procedure was applied to a coagulase negative culture filtrate. This material served as a control. The results of this experiment are presented in Table II. It can be seen that the three serum susceptible, coagulase negative, avirulent strains of *M. pyogenes* when inoculated into inhibitory serum which had been treated with the ammonium sulfate fraction from coagulase positive culture filtrates grew luxuriantly, while those inoculated into serum treated with the material from coagulase negative organisms failed to grow. Table III shows that the protective activity of the culture fraction from the coagulase positive strain is unaffected by heat, and therefore is suggestive evidence that the alpha toxin is not responsible for this protective effect. Further experiments in which purified preparations of alpha and beta toxin were added to serum confirmed this point, since no protective action of these preparations could be demonstrated.

The quantitative aspects of this protective mechanism of coagulase have not been thoroughly investigated as yet, although suggestive evidence indicates that a certain minimum amount of coagulase is necessary to protect a susceptible strain and induce it to grow in an otherwise inhibitory serum.

Two weakly coagulase positive strains,‡ which were nevertheless susceptible to serum bacteriostasis were inoculated into serum treated with 1.0 mg per ml of a coagulase preparation having a titer such that 1.0 mg of the dried preparation was necessary to produce a clot in 1.0 ml of plasma diluted 1:5 in 1 hour at 37°C. This represents a 100 times weaker preparation than that used in the previous experiment. Two coagulase negative strains were also inoculated into the serum thus treated and growth followed. The results of this experiment are presented in Table IV where it can be seen that the weakly coagulase positive strains were able to initiate growth while the coagulase negative strains were still inhibited.

TABLE III. Effect of Heat upon Protective Action of Ammonium Sulfate Precipitated Culture Filtrate Material.

Strain No.	Serum treated with *unheated* resist. strain filtrate ppt.* 0 hr	24 hr	Serum treated with *heated* resist. strain filtrate ppt. 0 hr	24 hr
1	4.5×10^2†	2.4×10^7	4.5×10^2	1.8×10^7
2	6.0×10^2	2.0×10^7	6.5×10^2	1.2×10^7
3	1.2×10^2	1.8×10^7	1.0×10^2	1.2×10^7

* & † Same as Table II.

‡ Undiluted filtrate from 24-hour broth culture gave a barely visible clot of 1:5 diluted plasma after 4 hours at 37°C followed by overnight incubation at room temperature.

TABLE IV. Growth of Weakly Coagulase Positive and Coagulase Negative Strains of *Micrococcus pyogenes* in Normal Human Serum Treated with a Weakly Active Coagulase Preparation.

Strain No.	Treated serum 0 hr	Treated serum 24 hr	Untreated serum 0 hr	Untreated serum 24 hr
1*	6.4×10^2	3.0×10^7	6.0×10^2	2.4×10^3
2*	5.3×10^2	4.5×10^7	5.5×10^2	7.5×10^2
3†	1.5×10^2	3.0×10^3	2.0×10^2	1.5×10^2
4†	2.1×10^2	"	2.4×10^2	1.0×10^2

* Weakly coagulase positive (see text).
† Coagulase negative.

Discussion. Although coagulase has been associated with the pathogenicity of staphylococci for many years, and is considered the one best criterion of pathogenicity(18), the mechanism of its action in the initiation of staphylococcal infections has not been clarified. Coagulase although tacitly assumed to function *in vivo* as it does *in vitro*, thus accounting for the characteristic localized lesions of staphyloccal infections, actually seldom does so. Fisher(19), even with the injection of massive doses of potent cultures and filtrates into rabbits was unable to show intravascular clotting either clinically, grossly, or microscopically, though controls of corresponding proportions of blood and staphylococcus culture in test tubes coagulated readily. Menkin and Walston(20) were also unable to produce lymphatic blockage in rabbits by the intracutaneous injection of active cell free coagulase. Trypan blue diffused readily from the site of its cutaneous inoculation, and the tributary lymphatics were found to be unoccluded.

It appears then that the *potential* pathogenicity of coagulase positive staphylococci might, in view of the findings reported here, be further strengthened by determining its ability to grow in normal human serum, since a certain minimum level of coagulase production appears necessary for this growth. Weak coagulase producers are inhibited by serum, and although considered pathogenic by most bacteriologists may, in fact, be incapable of initiating an infection. The possibility must be recognized that the actual factor responsible for growth of the staphylococci in serum is not coagulase but a substance associated with this substance.

Summary. 1. By following the growth of coagulase positive, virulent and coagulase negative, avirulent, strains of *M. pyogenes* in undiluted normal human serum for periods of 24 hours the coagulase positive strains grew very well while the coagulase negative strains were completely inhibited in their growth. Correlation of this differential growth of the 2 groups with any of the other common metabolic activities of this group of microorganisms could not be made. 2. It was postulated that coagulase might function in some way to protect the coagulase positive organisms against the antibacterial activity of human serum thus allowing them to grow. By treating actively bacteriostatic human serum with partially purified preparations of cell free coagulase, coagulase negative strains which were susceptible to serum bacteriostasis were able to grow. Alpha and beta toxins were ruled out as being responsible for this protective action, and the activity of the preparations in reversing the inhibitory action of serum was found to be heat stable, as is coagulase. 3. This finding suggests a new activity of coagulase which could conceivably function *in vivo* to aid the microorganisms in becoming established in the tissues of a host. With further quantitation of this reaction many weakly coagulase positive strains of *M. pyogenes* which are now classified as potentially pathogenic might be ruled out, since it appears that a certain minimal amount of coagulase is required to protect a strain against the antibacterial activity of human serum, and possibly also for it to initiate an infection.

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Isolation of Rabies Virus from an Insectivorous Bat (*Tadarida mexicana*) in California. (21725)

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In Florida during June, 1953, rabies virus was isolated from an insectivorous bat(1). This was the first isolation of the rabies virus from bats in the United States. In September of the same year the virus was also isolated from an insectivorous bat in Pennsylvania(2). These isolations prompted other groups to institute surveys and in November and December 1953, an isolation from a third area was made in Texas(3). Our isolation, the first from California bats, represents the fourth geographical area in the United States in which bats have been shown to harbor this virus. These isolations from a host of such myriad numbers and wide distribution suggest intriguing lines of investigation into the perplexing problems regarding rabies, especially the survival of the virus in nature. The findings of Pawan in Trinidad(4,5) that both the vampire bat and the fruit-eating bat might act as symptomless carriers for several months during which time their saliva was highly infectious lend emphasis to the importance of these isolations.

In July 1954, a survey of bats, in an attempt to isolate the rabies virus* was instituted in a 5 county area of California from which a high incidence of wild life rabies had been reported for the 4 previous years. For 1954, this area reported 6 foxes, 10 skunks, 1 wildcat, 1 raccoon, 3 cows, 1 horse, 1 sheep and 4 dogs as proven rabid. A total of 211 bats representing 14 species were collected, the brain and salivary glands of each bat were pooled, frozen and later inoculated into mice. This paper reports the isolation of the rabies virus from the tissues of Bat No. 68, a Mexican Freetail (*Tadarida mexicana*) collected on July 20, 1954.

Materials and methods. A 10% suspension of pooled brain and submaxillary salivary gland tissues of Bat No. 68 was prepared in physiological saline. Three hundredths ml of this suspension was inoculated intracerebrally into each of 6 adult Swiss mice of the Webster strain for the original isolation. Infectivity titration of this pool of brain and salivary gland tissue was by I.C. inoculation of 0.03 ml of log 10 dilutions from log 1 through log 12. Identification of the virus was by serum neutralization using Lederle's hyperimmune rabies antiserum and the method proscribed by the W.H.O. Committee on Rabies(6). Dilutions of the brain-salivary pool of 10^{-3} through 10^{-6} were used. A portion of the original 10^{-1} dilution of the tissues of Bat

* Supported by a grant from the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE I. Results of Inoculation of Tissues of Bat #68 I.C. in Mice.

Isolation						
Incubation period 5-6 days			Deaths 6/6* in 6-8 days			
Titration						
Dilution	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Deaths	6/6	5/6	6/6	6/6	0/6	0/6

Neutralization				
Dilution	Lederle's antisera	Termination day	Normal sera	Days incubation
10^{-3}	0/6	21	6/6	±10
10^{-4}	"	"	"	"
10^{-5}	"	"	"	"
10^{-6}	"	"	5/6	"

* Numerator is No. of mice that died; denominator is No. of mice inoculated.

No. 68 was submitted to Dr. Edwin H. Lennette and Dr. Harald N. Johnson for confirmation of identification. Intracranial and intramuscular inoculations of the original pool of tissues of Bat No. 68 were made into bats of 2 different species that had previously been held in captivity under observation for a period of 60 days. Subsequently inoculation of the brain tissue of these passage bats was made I.C. into mice.

Results. Table I details the pertinent isolation, titration and neutralization results. In the original isolation, the mice developed "explosive" symptoms on the 5th day and were all dead by the eighth day. The high titer (10^{-8}) of the original pool of tissues of Bat No. 68 has prompted close scrutiny and review of technics, but no errors have been elucidated. The neutralization was unequivocal and was confirmed by Drs. Lennette and Johnson by isolation, neutralization and the finding of Negri bodies in the first passage mice. Histopathological examination of the brain of Bat No. 68 revealed no Negri bodies, no neuronal damage and no evidence of an inflammatory reaction. Bat No. 68 was well-nourished and normal in appearance when trapped. Histopathological examination of the brain of first passage mice revealed numerous well-formed and characteristic Negri bodies with Sellers' stain and evidence of inclusions and encephalitis when stained with hematoxylin and eosin.

The results of intracerebral and intramuscular inoculation of two species of bats are detailed in Table II. These bats, Mexican Freetails (*Tadarida mexicana*) and Pallids (*Antrozous pallidus*), were held under observation for 60 days prior to inoculation. In each case the I.C. inoculated Freetails developed the furious type rabies while the Pallid became paralyzed. Incubation periods were very uniform. Interestingly enough the I. C. inoculated bats developed symptoms while none of those inoculated I. M. did. Histopathological examination of the brain of bats inoculated I.C. revealed Negri bodies and evidence of encephalitis. The brains of bats inoculated I.M. have not as yet been examined, or inoculated into mice.

Discussion. The primary significance of this isolation is the finding that California bats

TABLE II. Results of Inoculation of Bats with Tissues of Bat #68.

Species of bat		Route of inoculation	Incubation, days	Type of symptoms	Day of termination
Freetail	229	I.C.	15	Furious	Dead 17 days
	230	"	"	"	Sac. 18
	241	"	"	"	"
	242	"	"	"	"
	243	"	"	" & partial paralysis	"
	231	I.M.		None	Normal 45
	232	"		"	"
	233	"		"	"
Pallid	235	I.C.	16	Paralyzed	Dead 17
	236	"		None	Normal 50
	246	"		"	Dead 11
	237	I.M.		"	Normal 45
	238	"		"	"
	239	"		"	"

as well as those of Florida, Pennsylvania, Texas and Montana harbor the virus of rabies. (Subsequent to our isolation the virus was isolated from a bat in Montana(7), making the fifth widely separated geographical area in the United States.) This strongly suggests that the rabies virus is very widely disseminated in the insectivorous bats of the United States. The migrational habits of some of our bats bring them into contact with the Vampire Bat in Mexico.

What role this newly-recognized host plays in the transmission of the virus to new susceptibles or in enabling the virus to survive in nature remains to be investigated. It is interesting to note that none of the bats that were inoculated I.M. with the tissues of Bat No. 68 showed symptoms of rabies even though they were inoculated with the same material as those inoculated I.C. and that one I.C. inoculated bat failed to develop symptoms. The small numbers of bats inoculated plus the fact that their exposure history is not known relegates this to the position of an interesting finding only, although it certainly merits further investigation.

Summary. The virus of rabies has been isolated from a Mexican Freetail bat (*Tadarida mexicana*) collected in northern California July 20, 1954. The isolation of this virus and certain aspects of its passage history merit further investigation.

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Utilization of Dipeptides by Mammalian Cells in Tissue Culture. (21726)

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It has been shown in previous communications(1,2) that 12 amino acids, all in the L-configuration, are essential for the growth of both a mouse fibroblast (strain L) and a human carcinoma cell (strain HeLa) in tissue culture. As will be shown, each of 6 dipeptides tested has been found to substitute for the corresponding essential amino acid(s).

Methods. The methods of maintaining stock cultures, of setting up replicate experimental flasks, and of evaluating the growth response have been described in previous papers(1,2). The experimental medium consisted of the demonstrably essential amino acids and vitamins, salts, and accessory growth factors, supplemented with the minimum concentration of dialyzed horse or human serum found necessary to sustain growth of the mouse fibroblast and human HeLa cell, respectively (Table I).

Results. In the limiting medium of Table I, the cells proved capable of sustained growth and multiplication for many weeks, with an approximate generation time of 48 hours. The omission of a single amino acid from that medium led to the early degeneration and death of the culture. As illustrated in Fig. 1 and 2, a dipeptide containing the missing amino acid could substitute for it, and permitted growth and multiplication. In this manner, glycyl-L-leucine, glycyl-L-isoleucine,

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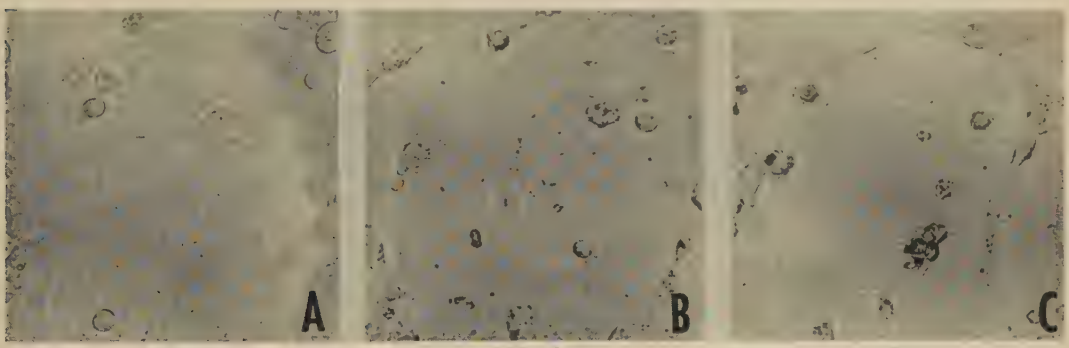
TABLE I. Media for Cultivation of HeLa Cell and Mouse Fibroblast.*

L-Amino acids (mM)		Vitamins (g/ml)		Salts (%)	
Arginine	.1	Biotin	10^{-6}	NaCl	.68
Cystine	.05 (.02)†	Choline	"	KCl	.04
Histidine	.05 (.02)	Folic acid	"	$NaH_2PO_4H_2O$	.014
Isoleucine	.2	Nicotinamide	"	$NaHCO_3$	.22
Leucine	.2 (.1)	Pantothenic acid	"	$CaCl_2$	.02
Lysine	.2 (.1)	Pyridoxal	"	$MgCl_2$	.008
Methionine	.05	Thiamin	"		
Phenylalanine	.1 (.05)	Riboflavin	10^{-7}	Misc.	
Threonine	.2 (.1)			Glucose	0.1 %
Tryptophan	.02 (.01)	Serum protein‡		L-Glutamine	1-2 mM
Tyrosine	.1			Penicillin	0.005 %
Valine	.2 (.1)			Streptomycin	"
				Phenol red	0.0005%

* After (1,2,3).

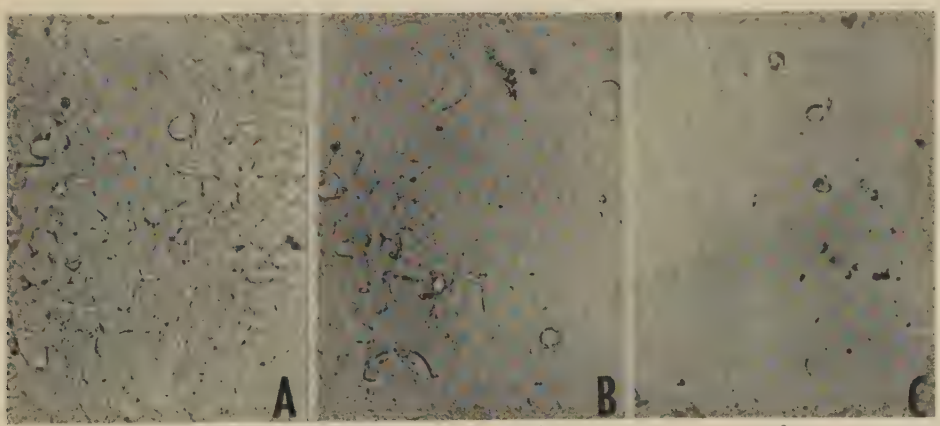
† For mouse fibroblast.

‡ In the present experiments, supplied as dialyzed human serum (5%) for the HeLa cell, and dialyzed horse serum (1%) for the mouse fibroblast. In the maintenance of stock cultures, whole human and horse serum were used, in concentrations of 10% and 5%, respectively.



0.1 mM 0.01 mM 0

FIG. 1. Growth response of HeLa cell to glycyl-DL-phenylalanine in a phenylalanine-deficient medium (magnification 120×). Cells were suspended in medium of Table I, but containing varying concentrations of glycyl-DL-phenylalanine in lieu of L-phenylalanine. Figures show the 6-day growth response to (A) 0.1 mM of the dipeptide, (B) 0.01 mM, and (C) to the control medium containing no added dipeptide.



L-Tyrosine 0.1 mM L-Leucyl-L-tyrosine 0.1 mM 0

FIG. 2. Growth response of HeLa cell to L-leucyl-L-tyrosine in a tyrosine-deficient medium (magnification 120×). Cells were suspended in medium of Table I, but containing varying concentrations of L-leucyl-L-tyrosine in lieu of L-tyrosine. Figures show the similarity in 6-day growth response to (A) 0.1 mM L-tyrosine and (B) 0.1 mM L-leucyl-L-tyrosine, and (C) absence of growth in a control medium containing no added dipeptide.

TABLE II. Growth Response of a Mouse Fibroblast (Strain L) to Dipeptides in Lieu of Essential Amino Acids.

Essential amino acid omitted from medium	Dipeptide added to medium	Inoculum × 10^4	Incubation time, days	Molar conc. of peptide: 2.0	0.1	0.02	0.005	0.001	0 (Degree of cellular multiplication*)	Conc. permitting max growth, mM: >	<	Optimal conc. of corresponding L-amino acid, mM
L-Phenylalanine	Glycyl-DL-phenylalanine	19	5	8.0	9.5	3.1	1.3	1.2	.8	.02	.1	.02
"	DL-Phenylalanylglycine	19	5	7.7	8.9	1.7	1.2	.74	.8	.02	.1	"
L-Leucine	Glycyl-L-leucine	22	5		10.1	4.9	3.4	3.1	2.4	.02	.1	.05
"	L-Leucylglycine	19	5	7.5	5.0				.5		.1	"
"	L-Leucyl-L-tyrosine	19	5		11.8	5.4	3.2	1.5	.8	.02	.1	"
L-Tyrosine	Glycyl-L-tyrosine	19	5	9.1	9.8	3.9	1.2	.42	.47	.02	.1	.02–.05
"	L-Leucyl-L-tyrosine	19	5	7.0	8.9	7.3	1.6	.9	.5	.005	.02	" – "

* Referred to inoculum as 1.

TABLE III. Growth Response of a Human Carcinoma Cell (Strain HeLa) to Dipeptides in Lieu of Essential Amino Acids.

Essential amino acid omitted from medium	Dipeptide added to medium	Inoculum × 10^4	Incubation time, days	Molar conc. of peptide: 0.5	0.1	0.03	0.01	0.003	0 (Degree of cellular multiplication*)	Conc. permitting max growth, mM: >	<	Optimal conc. of corresponding L-amino acid, mM
L-Phenylalanine	Glycyl-DL-phenylalanine	21	6	4.8	5.5	2.9	.7	.6	.5	.03	.1	.05
L-Isoleucine	Glycyl-L-isoleucine	8	6	3.6	5.4	5.7	2.3	1.1	.4	.01	.03	.1
L-Tyrosine	Glycyl-L-tyrosine	21	6	7.9	6.7	6.7	1.1	.7	.6	.01	.03	.03
"	L-Leucyl-L-tyrosine	21	6	6.4	7.0	6.4	2.1	.5	.5	.01	.03	.03
L-Leucine	L-Leucyl-L-tyrosine	21	6	5.0	7.0	3.8	1.1	1.0	1.0	.03	.1	.1

* Referred to inoculum as 1.

glycyl-DL-phenylalanine, and glycyl-L-tyrosine were found to substitute for L-leucine, L-isoleucine, L-phenylalanine, and L-tyrosine, respectively. Dipeptides with the essential amino acid as the acyl group (DL-phenylalanylglycine and L-leucylglycine) were similarly effective; and L-leucyl-L-tyrosine substituted for both leucine and tyrosine.

The quantitative aspects of the growth response to the dipeptides are shown in Tables II and III. With both the mouse fibroblast and the HeLa carcinoma cell, the molar concentrations of dipeptide necessary for maximal growth were of the same order of magnitude as those required of the simple amino acids.

Summary. Twelve amino acids had proved essential for the survival and growth of both the mouse fibroblast (strain L) and a human carcinoma cell (strain HeLa). In media lacking a single essential amino acid, in which the cells would otherwise degenerate and die, normal growth and multiplication were obtained on the addition of a dipeptide containing the essential amino acid. The effective concentrations of the dipeptide were of the same order of magnitude as those required of the simple amino acid. It remains to be seen whether the dipeptide is incorporated into cellular protein as such, or whether it is first split into its component amino acids(3).

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"Myocardial" Action and Standard Unitage (U.S.P. XIV) of Cardiac Glycosides.* (21727)

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A number of biological effects of the cardiac glycosides have been used for evaluation of therapeutic effectiveness. The assay methods in use, which determine the total dose required for cardiac arrest, are not always directly related to clinical effectiveness as reported by various authors(1,2). A number of investigators have tried to differentiate between the toxic dosage (basis of the assay methods) and specific cardiac activity. A great deal of study followed Hanzlik's proposal(3) that emetic action in the pigeon should be taken as a measure of cardiac activity and death as that of toxicity. While in general it has been debated(4) whether the emetic action, which has a multiple mechanism(5), is a measure of therapeutic effectiveness, it has been observed that the emesis/toxicity index changes according to the age of the pigeon(6). That the *relative* activity of cardiac glycosides may vary according to the biological method used is known from other examples; *e.g.,* the potency determined on the isolated mammalian or frog heart may deteriorate without a proportional alteration of toxicity(7).

It has been demonstrated experimentally (1,8-10) as well as clinically(11-14) that the cardiac glycosides exert a direct action on the myocardium. Hajdu and Szent-Györgyi(15, 16) have developed a method to register the *isometric* contraction of the frog heart; by decreasing the frequency of the electrical stimulation a "staircase" is formed, which is abolished by certain drugs (as well as by a decrease of K-ion concentration or a decrease in temperature). These authors consider that the amount of drug required to abolish the staircase represents its myocardial activity. They indicate that this action is independent of the direct contractile effect on the heart. The purpose of the present study is to correlate staircase-abolishing action with total *in vivo*

* Abstract presented at the fall meeting of the Am. Soc. for Pharmacol. and Exp. Therap., Charlottesville, Va., Sept. 7, 1954.

effects, and relate the findings to known clinical effects of the drugs in use.

Methods. For *in vivo* action we selected the pigeon-toxicity test, as recommended by the U.S.P. XIV(17). All samples were dissolved in 70% ethanol and then diluted with normal saline. Intravenous infusion was completed within 65-95 minutes (variations of infusion time affect the results). A total of 90 pigeon-toxicity tests were run on 10 samples. Our figures for the known samples are in complete agreement with the labeled amounts and the literature references.

The following cardiac drugs were investigated: 1 - 2 U.S.P. Reference Standard Digitoxin and Digitalis Powder. 3. Ouabain U.S.P. Powder (N. Y. Quinine and Chemical). 4. Gitalin NNR Amorphous (Gitaligin, White). 5-6. Fraction J. and S. prepared by Drs. Howard White and J. Dailey. 7. Digalen NNR 26% Alcohol Soln. (Roche). 8. Digilanid NNR in Soln. (Sandoz). 9. Scillaren NNR 25% Alcohol, 20% Glycerin Soln. (Sandoz). 10. Digoxin, U.S.P. 20% Alcohol Soln. (Burroughs Wellcome) Fractions J and S, prepared by Drs. White and Dailey by various fractionation procedures from digitalis powder, were selected from among a number of samples as the most active ones in the staircase-abolition test. Samples 1 to 6 were dissolved in propylene glycol and then diluted with saline, whereas samples 7 to 10 were diluted directly with saline. In no case did the concentration of glycol in the bath reach 0.5%, which *per se* has no effect on the heart. All solutions were freshly prepared in both types of experiments.

For the staircase abolition the procedure of Hajdu and Szent-Györgyi was closely followed (15). The frog heart was cauterized at the sino-atrial node to abolish its neurogenic rhythmicity. In each experiment the minimal amount of digitoxin, used as a standard, was compared with the amount of sample needed to maintain the amplitude of contraction when the frequency of stimulation was reduced from 15 to 6 per minute. We have found this particular frequency variation the most convenient for a comparison of drug effects. For instance, in 32 separate experiments on digitoxin we obtained a mean value of 2.75 γ (min. 2 γ, max. 4 γ) with a standard error of $\pm$ 0.052 γ, and P = 0.001, indicative of a high degree of reproducibility. The change in amplitude at higher frequencies (*e.g.*, from 30 to 15 per min.) is abolished in general with smaller amounts of drug, but the variability between experiments is much greater. On the other hand, when we attempted to abolish the difference of amplitude at lower frequencies (such as from 6 to 2 per min.), the appearance of "extrasystoles" and contractures interfered. The relatively high doses of glycosides required in this low frequency range may be responsible for these irregularities.

TABLE I. Pigeon Toxicity (U.S.P. XIV Unitage) and Activity on Staircase in Frog Hearts of Cardiac Drugs.

Drug	Frog heart, MED (γ)	Pigeon toxicity, MLD (mg/kg)	$\frac{\text{MED}}{\text{MLD}} \times 100$
Digitalis U.S.P.	2350	99.4	2.3
Digalen*	515 (.044 cc)	13.3 (1.17 cc)	3.85
Digilanid	64	.47	13.7
Fraction S	29.4	4.26†	.7
" J	25	2.28‡	1.1
Ouabain	6	.18	3.33
Gitalin	4	1.44	.28
Digitoxin U.S.P.	2.75	.52	.53
Digoxin	2.5	.40	.62
Scillaren	.65	.25	.26

* Wt based upon $CHCl_3$ extract.
† 24 pigeons.
‡ 6 " .

A total of 75 frog-heart preparations were used in these experiments during the last 2 years. Of these, about half were used for approximate evaluation of the active doses which were then measured with maximum accuracy on the rest. A minimum of 3 determinations was made for each sample. A frog heart, after proper washing, could be used for repeated determinations, since its sensitivity remained unchanged. In general, we used a heart for about 4 successive measurements, during a period of 6-8 hours.

Results. The minimal effective doses (MED) required for staircase abolition and the MLD for the pigeon, as well as the ratios conventionally expressed as "percentage" of (MLD) pigeon unitage needed to give the myocardial effect, are in Table I.

The MED on staircase for the tested drugs

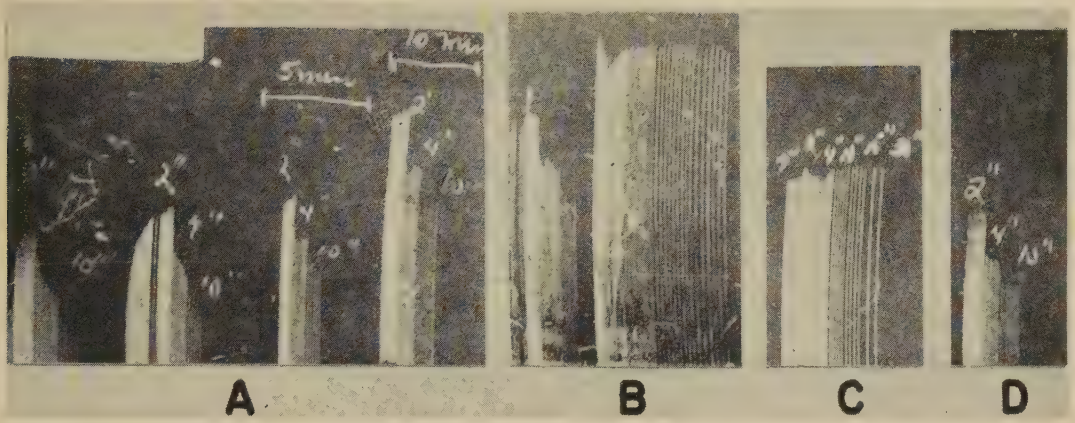

FIG. 1. A. Effect of 1 γ digitoxin on the staircase. First 2 sections show decreased contraction following stimulations of decreasing frequencies (30, 15 and 6/min.). In 3rd and 4th recordings, 5 and 10 min. after digitoxin application, amplitude is increased but staircase is not yet abolished. B. Complete abolition of the staircase with 2 γ digitoxin (1st recording, before treatment). C. Complete abolition of the staircase with 1 γ Scillaren. D. Normal staircase after washing out Scillaren.

varied from 0.65 to 2350 γ, while the MLD for the pigeon varied on a narrower range, from 0.18 to *ca.* 100 mg/kg. Correspondingly, there was a very wide variation in the activity/toxicity ratios.

The lack of relationship between pigeon unitage and staircase-abolishing potency among the various digitalis preparations is impressive. The fraction of the standard unitage required for staircase abolition varied from 0.7 to 13.7% for Fractions S and J, Digitalis U.S.P., Digalen and Digilanid, while for the purified glycosides Gitalin, Digitoxin and Digioxin, this fraction was contained between 0.28 and 0.62%.

On a weight basis, the highest myocardial activity was obtained with Scillaren and the glycosides Digoxin, Digitoxin, Gitalin and Ouabain.

Digitoxin and Digoxin remained close to each other in both actions and consequently the resulting ratios are also similar (0.53 and 0.62%), while Gitalin, though less effective on the weight basis, had a higher *relative* myocardial action (0.28% of the standard unitage). Digilanid gives the lowest *relative* staircase-abolishing effect: 13.7% of the pigeon toxicity, which on the other hand is rather elevated (0.47 mg/kg).

Ouabain, very active on the pigeon (0.18 mg/kg), gave as a corresponding ratio 3.33%. Scillaren had an MLD of 0.25 mg/kg, with a ratio of 0.26% for myocardial activity, *i.e.*, as high as that of Gitalin.

Discussion. These experiments reveal the independence of the standard (pigeon) unitage from another quantitative characteristic of the cardiac drugs, *i.e.*, their ability to maintain a higher work output under stimulations of decreasing frequency. Among 10 different glycosides, wide variation has been found between the minimal dose required for this effect and pigeon toxicity (U.S.P. XIV unitage).

The following examples suggest the specificity of the staircase-abolition test. Digoxin and Digilanid are equally effective on the heart-lung preparation according to Rothlin (18), and on pigeon toxicity according to our findings. In contrast to this, the staircase-abolition test revealed a 25-fold difference, corresponding to the findings on the isolated cat papillary muscle(19). Digitoxin and Digoxin were about equal in both the staircase-abolition and the toxicity tests, whereas K. K. Chen *et al.*(20) reported a 4-fold difference in their activity in causing systolic standstill in frogs.

In surveying the available comparative figures on cardiac activity in man of the drugs here considered, particularly the electrocardiographic determinations of Gold *et al.*(21,23), Batterman *et al.*(22) and DeGraff(2), we

could not find a definite correlation between stair-case-abolishing effect and clinical dosage. In certain instances, such as the lower effectiveness of Digitalis powder or Digilanid in comparison with Digitoxin, the displacement of clinical activity went in the same direction as myocardial action. This is in agreement with observations made by Cattell and Gold (19) for Digilanid, namely that its effectiveness in terms of unitage is lower on the isolated papillary cat muscle. On the other hand, lack of correlation is striking in the case of Scillaren(2), which has the same cardiac activity in man as Digitoxin although on the basis of its staircase-abolishing action it should be 4 times as effective.

This lack of correlation could be explained by assuming that direct myocardial action, even if it represents a specific physiological characteristic of the drug, is only one of the determining components *in vivo,* and that other features may well overshadow this effect on the isolated heart. For instance, it is known that the cardiac drugs have different metabolic rates (dissipation, absorption, elimination, accumulation), myocardial affinity, latent period and permeability(2,23-25). In this light perhaps the different ratios of toxicity—which alone is the resultant of central, vagal and cardiac effects—of staircase-abolishing activity and of the effect on the EKG may become explainable.

Summary. The ratio between standard pigeon unitage and dose required to abolish the staircase obtained by decreasing the frequency of electric stimulation on the isolated frog heart has been established for ten cardiac glycosides. The latter method, although it reveals a specific physiological characteristic independent of standard unitage, cannot be directly correlated with clinical effectiveness.

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Virus Absorption by Tumor Cells.* (21728)

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Transplanted tumors support the multiplication of a neurotropic variant of WS influenza virus (NWS) but resist infection by the non-neurotropic parent strain(1). Although these viruses have a common ancestor and are similar antigenically, they also differ markedly in certain other biological characteristics. Burnet(2) has shown that NWS virus, unlike all other influenza virus strains, cannot be converted to the "indicator" state and possesses an extremely heat labile hemagglutinin. But perhaps the most interesting evolutionary alteration in this influenza sport is its inability to hydrolyze the mucopolysaccharide substrates of influenza viruses. Due to this absence of enzymic activity, NWS virus shows little capacity for spontaneous elution from the surface of erythrocytes and other cells, although its adsorptive capacities are unimpaired. In studying tumor cell-virus interaction *in vitro,* Moore and Diamond(3) found that large doses of certain influenza or Newcastle disease virus strains, which are incapable of causing active tumor infections, inhibit the growth and transplantability of the Ehrlich ascites carcinoma and Sarcoma 180. Apparently this tumor-suppressive effect is not merely due to surface action of the virus, since normal tumor growth occurs after transplantation of cell suspensions from which the virus has eluted spontaneously or after treatment with receptor-destroying enzyme (RDE).

The present studies were undertaken to determine whether interaction of NWS virus and tumor cells differs from classical *in vitro* influenza virus-cell systems. In this way it was hoped that some light could be shed on the nature of tumor cell susceptibility to NWS virus infection.

Materials and methods. The murine ascites *tumors* used in these studies were obtained from Dr. Alice E. Moore of the Sloan-Kettering Institute for Cancer Research and were maintained in this laboratory by passage in male Swiss mice (Webster strain). The Ehrlich ascites carcinoma (EAC) was transferred at 7-8-day intervals by intraperitoneal inoculation of approximately 10^7 tumor cells. Sarcoma 180 (S-180) was usually passaged every 10 days, the 0.1-ml inocula containing approximately 10^6 cells. Ascitic fluids were harvested immediately prior to use and the supernatant fluids removed after centrifugation at 2,500 RPM for 20 minutes. The sedimented tumor cells were then suspended in buffered saline solution, washed 3 times and finally resuspended in a quantity of buffered saline solution sufficient to make a 12% tumor cell suspension. The cell counts of the final EAC preparations ranged between 15,500 and 18,600 tumor cells/mm^3; the S-180 cell counts were generally somewhat lower. *Chicken erythrocytes.* The plasma was removed from citrated chicken blood, the sedimented cells washed 3 times and 3% suspensions prepared by dilution with buffered saline solution. The red blood cell counts of these final suspensions were 83,000-85,000/mm^3; their absorptive capacity for NWS virus was approximately equivalent to the 12% tumor cell suspensions. *Viruses.* Allantoic fluid preparations of the WS strain of influenza A virus and its neurotropic substrain (NWS) were used in all studies. The NWS line(4) had undergone 23 consecutive egg passages and the WS strain 107. Fluids were harvested 44 hours after allantoic inoculation of a 10^{-3} dilution of seed material and stored at –20°C for not longer than 1 month prior to use. The NWS allantoic fluids were used undiluted; because the WS preparations invariably had a higher virus content they were diluted with normal allantoic fluid to adjust their hemagglutinin titers to the same levels as the NWS fluids. *Receptor-destroying enzyme (RDE).* Concentrated, purified RDE with receptor-

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TABLE I. HA Content of Supernatant Fluids after Incubation (37°C) of WS Virus with Erythrocytes and EAC Cells.

Time, min.	RBC		EAC		EAC*	
0	256		256		256	
15	4		64		0	
30	4		192		0†	
45	4		256		0	
60	3	+RDE‡	128	+RDE‡	0	+RDE‡
120	4	256	128	128	0	192
180	4	128	128	192	0	192

* Virus heated 56°C for 30 min.
† <2 (reciprocal).
‡ 500 units at 105 min.

destroying activity(5) of 50,000 units/ml was prepared by Dr. J. F. McCrea from the 4Z strain of *Vibrio cholerae*. The viral hemagglutinin content of fluids containing RDE was determined in the presence of 2% sodium citrate. *Buffered saline.* All reactions were carried out at pH 6.1-6.2 in isotonic sodium chloride solution containing 0.5 M sodium acetate-acetic acid buffer and 0.1% calcium chloride. This menstruum was chosen to provide optimal conditions for the action of RDE (5) as well as the "enzymes" of the WS viruses(6). *Hemagglutination titrations.* The hemagglutinin (HA) content of the various fluids was assayed by the pattern test using 0.5% chicken erythrocytes in a final volume of 0.5 ml. End points were taken as the last tube showing partial (50%) agglutination after 30 minutes at room temperature. *Adsorption-elution tests.* A series of tubes containing 1.0 ml each of the washed cell suspension and virus were incubated with frequent agitation in constant temperature water baths. At various intervals the virus-cell mixtures were centrifuged at 2,500 RPM for 5 minutes and an aliquot of supernatant fluid tested for HA content. In order to determine the amount of adsorbed, elutible virus, 0.1 ml of RDE containing 500 receptor-destroying units was added to the last tubes in each series, generally after 105 minutes of incubation. Treatment of virus-cell mixtures with RDE was always carried out at 37°C and supernatant fluid HA titers determined in duplicate after additional incubation periods of 15 and 75 minutes. Control virus-cell suspensions were treated in the same manner with 0.1 ml of buffered saline.

Results. WS virus adsorption and elution. A comparison of WS virus absorption by chicken erythrocytes and EAC cells is shown in Table I. Peak absorption of virus was noted in 15 minutes and constant HA levels of the supernatant fluid were maintained for the ensuing 3 hours. The addition of RDE resulted in complete elution of the virus. On the other hand, only a slight, transient decline in the HA titer of fresh WS allantoic fluid occurred with 6% EAC cells (final concentration). Preparations of WS virus that had been stored at –20°C for several months were more readily absorbed by EAC cells than fresh preparations. However, WS virus could be made to adhere more firmly to EAC cells by preliminary heating of the allantoic fluid at 56°C for 30 minutes, thus destroying its infectivity and enzymic activity. Under these conditions no HA was detected in the supernatant fluids in 15 minutes or up to 3 hours after incubation of inactivated virus with EAC cells. Complete liberation of heated-virus HA could be achieved by treatment with RDE.

NWS virus adsorption and elution. As noted in Table II, the red blood cell suspension completely adsorbed 256 HA units of live NWS virus. No spontaneous elution occurred in the 3-hour experimental period but the virus could be completely liberated by treatment with RDE. In striking contrast to their inability to absorb infective WS virus, EAC cells effectively removed all NWS virus HA from the supernatant fluids. S-180 cells exhibited similar absorptive properties. Also shown in Table II is the effect of RDE on the tumor cell-virus complexes after 37°C incuba-

TABLE II. HA Content of Supernatant Fluids after Incubation (37°C) of NWS Virus with Erythrocytes and Tumor Cells.

Time, min.	RBC		EAC		S-180	
0	256		256		256	
15	2		3		0	
30	0*		2		0	
45	0		0		0	
60	0	+RDE†	0	+RDE†	0	+RDE†
120	0	128	0	0	0	0
180	0	192	0	0	0	0

* <2 (reciprocal).
† 500 units at 105 min.

tion for 105 minutes. NWS virus bound by EAC or S-180 cells could not be eluted by receptor destruction. These experiments were repeated numerous times with different NWS virus and tumor cell preparations and with concentrations of RDE up to 50,000 units, always with similar results. It appears, therefore, that tumor cells are capable of "fixing" NWS but not WS virus hemagglutinin.

Destruction of tumor cell receptors by RDE and periodate. Erythrocytes exposed to RDE(5) or sodium periodate(7) lose their capacity to absorb influenza virus. Tumor cell receptors for NWS virus were also found to be susceptible to these agents, indicating an essential similarity in the absorptive mechanisms of tumor cells and red blood cells.

EAC cells were exposed to 500 units of RDE for 30 minutes at 37°C, washed 3 times in citrate-saline and resuspended in acetate-buffered saline. When a 12% suspension of these cells was mixed with an equal volume of NWS allantoic fluid (HA titer 1:256) at 37°C, no decrease in the HA content of the supernatant fluid occurred in 3 hours. The receptor-destroying activity of periodate ion was tested by suspending washed EAC cells and chicken erythrocytes in 0.001 M sodium periodate in isotonic saline for 60 minutes at room temperature. Erythrocyte and EAC cell suspensions treated with sodium periodate in the same manner in the presence of 5% glucose(7) served as controls. The 2 tumor cell and 2 red blood cell preparations were then washed 3 times, suspended in acetate-buffered saline and tested for their capacity to absorb 256 HA units of NWS virus at 37°C. The results showed that sodium periodate (in the absence of glucose) destroyed the erythrocyte and tumor cell receptors, most of the virus HA remained in the supernatant fluids over the 3-hour experimental period. The absorptive properties of the control cell suspensions (treated with sodium periodate in the presence of glucose) were unaffected.

NWS virus adherence to tumor cells at various temperatures. The foregoing experiments were all carried out at a temperature of 37°C. At 23°C and 1°C rapid absorption of NWS virus on EAC cells was again noted but a much looser union was formed, resembling

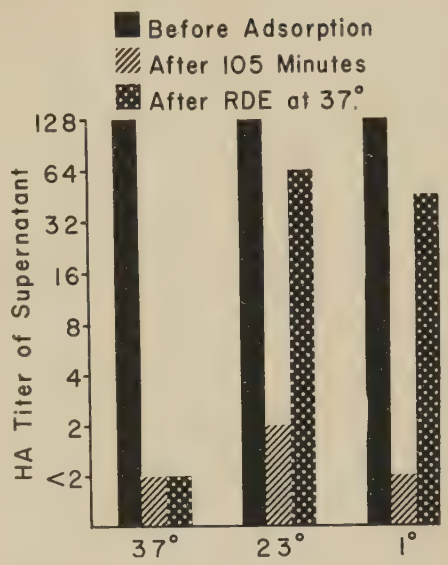

FIG. 1. Liberation of NWS virus from EAC cells after adsorption at various temperatures. Virus-cell mixtures were incubated for 105 min. prior to adding 500 units of RDE at 37°C.

the NWS virus-erythrocyte complex. When 500 units of RDE were added to mixtures of NWS virus and tumor cells after 105 minutes incubation at the lower temperatures a considerable portion of the adsorbed HA eluted into the supernatant fluids, whereas no HA could be liberated from EAC cells after virus adsorption at 37°C. (Fig. 1).

Effect of incubation time on the virus-binding capacity of EAC cells. NWS allantoic fluid (HA titer 1:256) was added to an equal volume of 12% EAC cells, the virus-cell mixtures distributed in a series of duplicate tubes and incubated at 37°C. At 30-minute intervals aliquot samples of supernatant fluid were withdrawn and tested for HA content. At corresponding intervals 500 units of RDE were added to each tube and supernatant fluid HA titers again determined after an additional 30-minute period of incubation with the enzyme. The curve in Fig. 2 shows that NWS virus was rapidly absorbed by EAC cells and no spontaneous elution occurred in 2½ hours. The effect of RDE on the virus-cell complex is shown by the broken lines extending from each point on the adsorption curve. As illustrated, much of the virus HA was liberated from the tumor cells in 30 minutes, but with increasing duration of virus-cell interaction

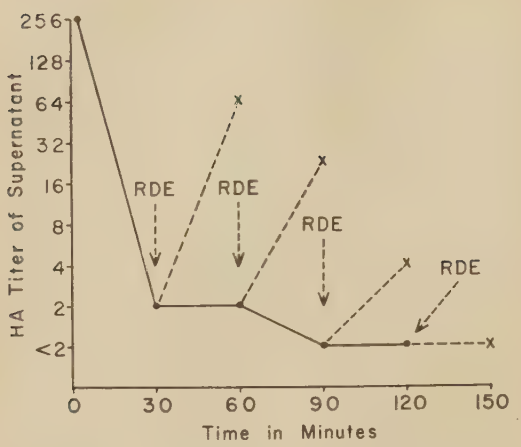

FIG. 2. NWS virus "fixation" by EAC cells as a function of duration of interaction at 37°C. Note the inability of RDE to liberate virus from the tumor cells after prolonged incubation.

there was a gradual diminution in the capacity of RDE to elute the virus and by 2 hours the enzyme was completely ineffective. NWS virus controls incubated at 37°C for 2½ hours in buffered saline solution or cell-free ascitic fluid exhibited no decrease in HA titer. It appears, therefore, that the degree of NWS virus adherence increases with time eventually leading to the formation of an indissociable tumor cell-virus complex.

Heated tumor cells. In order to determine whether tumor cells play an active role in "fixing" adsorbed virus, the capacity of RDE to dissociate virus from previously heated tumor cells was tested. One portion of an EAC cell suspension was incubated at 56°C for 30 minutes and another kept at room temperature. After adding NWS allantoic fluid, both samples were transferred to the 37°C water bath for 105 minutes and then treated with 500 units of RDE. Table III shows that NWS virus HA was completely absorbed by both the heated and unheated cell suspensions and no spontaneous elution occurred. Although it was again noted that RDE had no effect on virus adsorbed on unheated tumor cells, a considerable amount of HA was eluted from the heated tumor cells by enzyme treatment. These results suggest that "viable" tumor cells are essential for demonstrating the virus adherence phenomenon.

TABLE III. Effect of RDE on NWS Virus HA Adsorbed on Heated (56°C) and Unheated EAC Cells.

Time, min.	Unheated EAC		Heated EAC	
0	128		128	
15	0*		0	
30	0		0	
45	0		0	
60	0	+RDE†	0	+RDE†
120	0	0	0	48
180	0	0	0	64

* <2 (reciprocal HA titer supernatant fluid).
† 500 units at 105 min.

Discussion. The first step in cellular parasitization by influenza virus is adsorption to the receptors of susceptible cells, but the mechanisms by which virus penetrates the cell membrane are unknown. The former widely accepted view that the cell membrane is rendered permeable by the action of virus receptor-destroying enzyme was based largely on experiments with "non-living" substrates such as erythrocytes and excised mouse lung. Under these conditions influenza virus adsorption is usually followed by spontaneous elution, a process which can be accelerated and brought to completion by cholera RDE. However, erythrocyte absorption of the closely allied Newcastle disease virus (with low enzymic activity) is only partially reversible by RDE(8) and when certain influenza viruses are converted to the "indicator" state, they can be fixed to chicken erythrocytes *at a temperature of* 37°C(9). In addition, periodate-treated virus forms a firm union with erythrocytes that is unaffected by RDE(10). Fazekas de St. Groth has also presented evidence that red blood cells, excised mouse lung and chick allantoic cells exposed to appropriate amounts of periodate form an irreversible receptor-virus combination. Apparently, passive ingestion of active or heat-killed virus can be accomplished by living but not by formalinized allantoic cells, a process that has been dubbed "viropexis," suggesting that "penetration by the virus of the cell membrane does not depend on the viability or enzymic activity of the infective particle, but only on the functional integrity of the host cell"(11). An admirable analysis of the role of the virus in initiating cellular infection has been presented by Burnet(12).

The present studies demonstrated that in-

teraction of infective NWS influenza virus and Ehrlich tumor cells gradually resulted in cellular "fixation" of virus hemagglutinin, an effect which was found to occur at 37°C but not at room temperature or 1°C. Chicken erythrocytes or heated tumor cells were incapable of forming a firm union with the virus. It should be noted, however, that there is no evidence that NWS virus "entered" the intact tumor cells *in vitro*. Rather it is equally conceivable that an irreversible virus-receptor union gradually took place at the cell surface, resembling in certain respects the reactions of Newcastle disease virus or "indicator" and periodate-treated influenza virus with chicken erythrocytes. In this regard, it is of interest to recall that NWS virus also has little enzymic activity, an adaptive change in this mutant which possesses a broad spectrum of tissue infectivity, including the Ehrlich ascites tumor(13). The enzymically active WS parent strain, on the other hand, is incapable of multiplying in neoplastic tissue and does not form an indissociable union with Ehrlich tumor cells even after its capacity to elute has been destroyed by heating.

This unusual *in vitro* interaction of NWS virus and tumor cells, both presumably in the "living" state, may provide additional information on the nature of tissue susceptibility to influenza virus infection. Further studies are in progress.

Summary. Fresh preparations of the WS strain of influenza A virus were poorly absorbed by Ehrlich tumor cells but heated WS virus formed a loose union dissociable by treatment with RDE. The neurotropic variant of this virus (NWS), which is capable of multiplying in transplanted Ehrlich ascites tumor, readily combined with EAC cells and did not elute spontaneously. RDE and sodium periodate destroyed the tumor cell receptors of NWS virus. However, NWS virus hemagglutinin could not be liberated by RDE after interaction with intact EAC cells at 37°C for 105 minutes. This "fixation" of NWS virus was not demonstrable after shorter periods of incubation, at temperatures of 23°C and 1°C, or if the tumor cells were previously heated to 56°C.

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Comparison of Bacteria Isolated from Blood, Tissues and Feces of X-irradiated Mice.* (21729)

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(Introduced by J. Walter Wilson.)
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Many workers have isolated bacteria from the blood and organs of laboratory animals irradiated with an approximately midlethal dose of roentgen rays(2,3,5,7). Because most of the bacteria found are species normally encountered in the intestinal tract, it has been assumed that an irradiated animal's

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own gut is the source of these organisms. In all cases involving non-pathogens, evidence of their enteric origin was based on the familiar biochemical tests used for the recognition of bacterial species. Though the exact source of the organisms may be implied by such recognition, it is not proven. The experiments to be described were initiated to confirm (or deny) that the gut is the source of post-irradiation infection by making a serological comparison of cultures of a given species of organism isolated from the feces, and from the blood, the heart, or the spleen of individual irradiated animals.

The inbred BUB strain mice used in the present investigation have shown a high degree of qualitative stability in regard to certain types of bacteria in their fecal flora. A total of 1570 isolates of *Proteus mirabilis* from the feces of 820 animals (1 to 145 cultures from any single mouse) were invariably one of two serological types. These were designated serotype "A" or serotype "D" by means of tube agglutination tests of all the cultures, and serum adsorption tests with 597 (38%). In addition, 17 strains of *Proteus* isolated from bacteremias in irradiated BUB mice were shown by agglutination and adsorption tests to be *P. mirabilis* of either the "A" or "D" type(6). Further work with *P. mirabilis* and similar, though less extensive, studies made of *Paracolobactrum coliforme* and *Pseudomonas* isolated from BUB mice are reported below.

Exp. I. Twenty male BUB mice 60 to 90 days old were selected, 10 of which carried *P. mirabilis* while the other 10 were *Proteus* negative on the basis of 3 consecutive samplings of the feces. All these mice had *Pa. coliforme* in their feces and were free of *Escherichia coli* throughout the experimental period. Other laboratories have reported a high incidence of X-ray induced bacteremias with paracolons although the species have not been specified (5). Eosin methylene blue agar was used for the isolation and enumeration of paracolons and Salmonella-Shigella agar for *Proteus*. All cultures of *P. mirabilis* were typed by the macroscopic tube agglutination test in "A" and "D" antisera. All animals were individually housed and fed a diet containing 18% casein, 41% corn starch, 26% fat (20% crisco, 4% corn oil, 2% cod liver oil), salts, and yeast. After being on this diet for 2 weeks, the mice were irradiated with 600 r from a Picker-Waite X-ray Therapy machine. Physical factors: 200 kv, 20 ma, at a target distance of 20 cm; 3 animals at a time in styron containers placed in a wooden phantom for maximum back scatter. One mm Al and 0.5 mm Cu filters were used. The dose rate was 240 r/minute. The 30 day mortality for these mice at 600 r is about 75%. Daily tail blood samples taken from the irradiated animals from the 7th to the 26th day post irradiation were inoculated into thioglycollate and trypticase-soy broths. The tail of each mouse was bathed in tincture of metaphen for one minute before being snipped with sterile scissors. The blood was collected with a sterile capillary pipette. As a control, 16 unirradiated animals were bled similarly for 13 days and 8 additional mice were bled without antiseptic treatment of their tails. No growth was observed in the 208 samples from the unirradiated disinfected group. Of 104 blood samples from the untreated group, only two showed bacterial growth (*Micrococcus sp*). These results indicate that the blood samples were reliably free from contamination by bacteria on the tail surface. Irradiated animals that died were wrapped in aluminum foil and frozen in a deep freeze (–30°C) until such time as they could be autopsied(4). Heart and spleen were then ground in a Waring blendor with Trypticase-soy broth as a diluent. Aliquots of the homogenate were inoculated into tubes of Trypticase-soy broth. These broth tubes, as well as a portion of the original homogenate, were incubated for 48 hours at 37°C. At this time, any of the material manifesting growth was plated on appropriate mediums and isolates secured for examination. The organs of 35 normal mice that were sacrificed and frozen were free of the bacterial species being investigated in these organs—even though some of the animals had been permitted to remain at room temperature for periods up to 8 hours prior to freezing. Fecal samples taken daily from the 7th to the 26th day post irradiation from the 20 irradiated animals were diluted and plated on E. M. B. and S. S. mediums.

TABLE I. Organisms Found in Blood Cultures Taken Post-Irradiation and Organ Cultures at Autopsy.

	Mouse	Days post X-ray and bacteremias found																			Source of cultures at autopsy	
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Heart	Spleen
Proteus "positive" group	Q -69	—	—	—	—	—	—	—	—	M	St	—	—	—	—	—	—	—	—	—	—	(Animal survived, organs not cultured)	
	-70	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*Idem*	
	AJ -65	—	—	—	—	—	P C	P	P 2C	D												P	sterile
	-66	—	—	—	—	—	—	—	D													P	Ps
	-68	—	P	—	—	—	—	—	D													P, Ps	P, M
	AQ -80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(Animal survived, organs not cultured)	
	-83	—	—	—	—	—	M	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*Idem*	
	AW-79	—	—	—	—	—	M	— D														Ps, M	sterile
	-80	—	—	—	—	—	D															Ps, P	Ps
	-81	—	Pa	—	—	—	—	—	—	— D												P, Pc	M
Proteus "negative" group	T -28	—	—	—	—	M	M	—	D													Ps	Ps
	-29	—	—	Pc	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(Animal survived, organs not cultured)	
	Z -87	—	—	—	—	—	—	—	—	—	—	—	Ps	Ps	D							Ps	decomposed
	-88	—	—	—	—	—	Pa Ps	P Ps	P Ps	Ps	D											Ps	Ps
	-89	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(Animal survived, organs not cultured)	
	AJ -69	—	Ps	—	M	Ps D																Ps, Aa	Ps, M, Aa
	AN -73	—	—	—	—	—	—	M	M	—	Ps	Ps	D									Ps	sterile
	-75	—	—	—	—	—	—	—	—	—	—	—	—	—	—	M	—	—	—	—	—	(Animal survived, organs not cultured)	
	-78	—	—	—	—	—	—	—	—	—	—	M	—	—	—	—	—	—	—	—	—	*Idem*	
	AQ -81	—	—	—	— D																	sterile	sterile

Aa = *Aerobacter aerogenes;* C = unidentified Gram positive cocci; M = micrococci; P = *Proteus mirabilis* "D"; Pa = *Paracolobactrum aerogenoides*; Pc = *Paracolobactrum coliforme*; Ps = *Pseudomonas sp.*; St = *Streptococcus*; D = Death of animal; — = Bacteria not found in blood sample.

This was done to follow the numbers of *Proteus* and paracolon organisms in the feces and secure isolates for comparison with those encountered in the blood and organs. *Pseudomonas* was not sought systematically in the feces because it occurred infrequently in detectable numbers.

Proteus mirabilis. In the group of 10 animals positive for *Proteus*, 4 had *P. mirabilis "A"* consistently in their feces. All 4 survived and no *Proteus* was isolated from the tail blood samples (Table I). The remaining 6 harbored serotype "D". All 6 died with 2/6 having bacteremias and 5/6 having positive organ cultures for this serotype. The type "D" population rose in the feces following irradiation of the animals, whereas the type "A" fell during the period of maximuum appearance of bacteremias. This finding is consistent with the correlation between high bacteremia incidence and high fecal numbers of *Proteus* reported by this laboratory(1,6). It must be pointed out, however, that *Pseudomonas* was found on autopsy in 4 of these mice and may have played a role in their death. In the group of 10 animals originally negative for *Proteus*, several were observed to have this organism present in their feces following irradiation. Three mice yielded *P. mirabilis* at a low level on one day and a fourth animal carried it for 5 consecutive days. The latter animal, number Z-88, developed a *Proteus* bacteremia which was cleared and replaced with *Pseudomonas* (Table I). No other animal in this group had a *Proteus* bacteremia, nor was this organism isolated from heart blood or spleen on autopsy. Since an increase in *Proteus* level in the feces may result from irradiation of BUB mice(1,6), possibly some of the animals were carrying the organism at subdetectable levels prior to irradiation. In all cases, the *Proteus* was serotype "D". *Paracolobactrum coliforme.* A bacteremia appeared in which this organism was isolated in pure culture (mouse #T-29).† A rabbit was immunized with this strain and 285 *Pa. coliforme* fecal isolates including 29 from T-29 were tested by tube agglutination in this antiserum. All agglutinated to the titer of the homologous organism. Fifty-five (19%) were used in an adsorption study. All removed the homologous *Pa. coliforme* agglutinins from T-29 antiserum. At the time of autopsy, this species was found in the heart of another animal. Adsorption proved that it was serologically identical with the other paracolons so tested. The numbers of *Pa. coliforme* did not change significantly in the feces following X-irradiation of the animals. *Pseudomonas aeruginosa.* Four out of the 20 mice developed a *Pseudomonas* bacteremia. All 4 animals succumbed and the organism was recovered from their heart blood or spleen. Five of the remaining mice, for a total of 9/20, had *Pseudomonas* in their organs on autopsy. All these bacteria were considered *Ps. aeruginosa* on the basis of biochemical tests. Unfortunately, very few fecal isolates of these bacteria were available; but in 2 instances, a complete series could be assembled for comparison, *i.e.*, cultures from feces and blood of the living animal and an organ at autopsy. Such a series of isolates was obtained from mouse Z-88. An antiserum was prepared against the *Pseudomonas* culture from the initial bacteremia that appeared in this animal. All the *Ps. aeruginosa* isolates from mouse Z-88 agglutinated in the antiserum. Furthermore, the homologous agglutinins were removed by adsorption with representative cultures from the feces, the blood, and an organ (the heart) of animal Z-88. Tests in another *Pseudomonas* antiserum (designated "Ps op") from the laboratory stock collection‡ yielded essentially the same results. Isolates from a second animal, AN-73, from which another complete series was available, removed their own agglutinins from "Ps op" serum but not those for the Z-88 cultures.

Exp. II. Twenty-eight mice were selected by fecal flora screening and divided into 3 groups. One group of 10 were carriers of *P. mirabilis "A"* (called Group "A"), a second

† In biochemical reactions, it resembled the description in Bergey's Manual of *Escherichia coli acidilactici* except that 7 days were required for fermentation of lactose.

‡ Kindly supplied by Dr. C. A. Stuart. This serum agglutinated many more of the *Pseudomonas* isolates than Z-88 serum.

group of 9 were carriers of *P. mirabilis "D"* (Group "D"), and a third group of 9 apparently were free of *Proteus* (Group "O"). These mice were irradiated with 600r and kept under the same conditions as described for the previous experiment. No blood samples were taken. As soon as possible after death, the animals were frozen and later autopsied. Liver and kidney were ground and cultured as well as heart and spleen. *Proteus* isolates in this experiment were typed by slide agglutination using 1:8 dilutions of *P. mirabilis "A"* and *"D"* antisera.

Results. In Group A, 5 out of 10 animals were positive for *Proteus A* in one or more of their organs at autopsy. In Group D, 5 out of 9 animals had *Proteus D* in one or more organs. In Group O, no *Proteus* was isolated at autopsy. Thus, in every case the serotype of *P. mirabilis* isolated at autopsy conformed to the type present in the feces.

Discussion. Evidence available in the literature attests to the enteric origin of many of the bacteria isolated from radiation induced infections of laboratory animals. The results of the investigations described in this paper support the fundamental concept that the irradiated animal's own gut is the source of these organisms. Mice which carried *P. mirabilis* of either of 2 serotypes in their feces were largely consistent in having the same type in their blood, whether isolated before or after death. In order to show that these observations might not be restricted to *Proteus* alone, animals were selected in the first experiment which also carried *Pa. coliforme.* Although this organism invaded in only 2 instances, the isolates obtained in these instances were serologically identical with the *Pa. coliforme* in the feces of this particular group of mice.

In the work involving *P. mirabilis* and *Pa. coliforme,* groups of animals with known specific floral populations of these organisms showed that invasion by one of the species was confined in general to the type indigenous to each group. It was possible on a limited scale to demonstrate a similar consistency from one individual animal to another with infections caused by different strains of *Pseudomonas.* Isolates of *Ps. aeruginosa* from the feces, the blood and the heart of one mouse were serologically identical. An analogous series of isolates from another animal were not identical to that of the first animal.

Summary. A serological comparison of bacteria isolated from the blood, tissues and feces of X-irradiated mice has been made. Three species of organisms were studied in this manner: *Proteus mirabilis, Paracolobactrum coliforme* and *Pseudomonas aeruginosa.* The results indicate that the organisms found in the blood and organs of the X-irradiated mice were serologically identical to bacteria of the same species found in the feces.

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Possible Mechanism of Urea Toxicity in Ruminants.*† (21730)

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The urea toxicity syndrome in lambs has been described by Repp *et al.*(1), and is characterized by the following clinical symptoms in order of their appearance; restlessness, ataxia, labored breathing, collapse, muscular spasms, tetany, and death. This toxic action of urea is generally considered to be the result of a rapid liberation of ammonia from this compound in the rumen, and subsequent absorption of the ammonia through the rumen wall. Repp *et al.*(1) have shown a close correlation between the levels of blood ammonia and the degree of urea toxicity observed in sheep. Clark *et al.*(2) failed to produce typical urea toxicity symptoms by intravenous injections of ammonia solution in this species. However, their data suggest that some toxic compound is produced by the urea in the rumen resulting in the death of the animal. It has been reported by Kaishio *et al.*(3) that urea is converted to ammonium carbamate in the rumen or abomasum, and that toxicity is the result of the absorption of this compound. The direct conversion of urea to ammonium carbamate does not seem likely due to the high urease activity found in the rumen, and, in addition, several non-protein nitrogen compounds other than urea will cause the typical urea toxicity syndrome(1).

The purpose of this investigation was to study the role of ammonium carbamate in relation to non-protein nitrogen toxicity in ruminants.

Methods and materials. Thirteen ruminating lambs ranging from about 7-12 months of age were used in this experiment. The general procedure was to inject intravenously into lambs varying amounts of ammonium carbamate and observe toxicity symptoms. The ammonium carbamate, $NH_2CO \cdot ONH_4$, was prepared by mixing anhydrous ammonia and carbon dioxide in cold absolute ethanol. The crystals were filtered, dried on the funnel by suction, then stored in a refrigerator until ready for use. Lambs were also given ammonium carbamate by drench and by injection into the abomasum. Control lambs were injected with urea and ammonium chloride. All intravenous injections were given via the jugular vein. For injection, the carbamate and urea were dissolved in 10-12 ml of distilled water, and the ammonium chloride in 25 ml of physiological saline. When administered by drench, 100 ml of solution were given, and 50 ml when injected into the abomasum. Blood samples were taken from the jugular vein for ammonia determinations just prior to administration of the test material and after administration whenever symptoms of toxicity appeared. When no symptoms appeared, blood was taken at about 15 minutes after injection. Blood ammonia was determined by the method of Metzler(4).

Results. The results of the experimental treatments are shown in Table I. As increasing amounts of ammonium carbamate were injected the degree of toxicity in the animal was increased with death resulting at the 6 g level. Toxicity symptoms observed appeared to be of the same type as those reported for urea(1). Introduction of ammonium carbamate into either the abomasum or rumen proved toxic. Injection of ammonium chloride or urea failed to produce ammonium carbamate toxicity symptoms. In no instance was there an appreciable rise in blood ammonia.

Discussion. Intravenous injections of ammonium carbamate produced typical urea toxicity symptoms in lambs. Oral administration of the compound, or injections into the abomasum, produced similar symptoms. These data suggest that urea toxicity in ruminants is not due to an ammonia toxicity *per se* even though very high blood ammonia values may be observed during toxicity symp-

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TABLE I. Effect of Intravenously Injected and Orally Administered Ammonium Carbamate, Urea, and Ammonium Chloride on Lambs.

Wt, lb	Compound and method of administration		Response of lamb	Blood NH_3-N Before	Blood NH_3-N After
	g			μg/100 ml	
121	2 $NH_2CO \cdot ONH_4$	Intrav.	Mild A* for 5 min., R*	445	446
132	3		Moderate A, dyspnea, R	147	257
115	4		Severe A, dyspnea, collapsed, R	469	576
113	5		A, dyspnea, collapsed, tetany, R	219	229
121	6		A, collapsed, severe convulsions, tetany, died		
70	20	Inj. into abomasum	A, collapsed in 20 min., tetany, death in 25 min.		
70	25	*Idem*, just after feeding	Severe A, muscular spasms, R in 1 hr		
64	15	*Idem*	Mild A, recovered in 35 min.	623	566
64	25	Rumen by drench	A, collapsed, tetany, death in 10 min.	421	781
70	2 NH_4Cl	Intrav.	Dizziness, lethargy, R in 30 min.	438	495
102	5 $NH_2CO \cdot NH_2$	"	No effect	428	424
60	15	Inj. into abomasum	*Idem*		
100	7 $NH_2CO \cdot ONH_4$	Subcut.	"		

* A = Ataxia; R = Recovered.

toms. Upon the injection of ammonium chloride, complete relaxation of the skeletal muscles was observed in contrast with muscular spasms and tetany observed in urea or ammonium carbamate toxicity. Due to fermentation in the rumen, large amounts of carbon dioxide are produced. It is known that in the presence of ammonia the bicarbonate ion exists in equilibrium with the carbamate ion(5). Therefore, due to the rapid liberation of ammonia from urea in the rumen, relatively large concentrations of ammonium carbamate may be present. This compound is apparently rapidly absorbed as toxicity symptoms appeared about 2 minutes after oral administration.

Repp *et al.*(1) were able to reverse a urea toxicity in lambs by oral administration of acetic acid provided the animals had not reached the state of tetany. No explanation was available for the prophylactic action of acetic acid. However, ammonium carbamate is decomposed instantly in a weak acid solution to ammonia and carbon dioxide(5), and in view of the findings presented in this report it appears likely that acetic acid exerted its beneficial action by the above mechanism.

When urea was administered orally in doses that were fatal or near fatal, bloating was observed(1). Conditions in the rumen at this time may be similar to those when ruminants suffer from acute bloat on immature legume pasture. Immature legumes are high in nonprotein nitrogen(6) and are readily fermentable in the rumen. This would favor the high production of ammonia and carbon dioxide. In consideration of results in this experiment it appears plausible that death during acute bloat in cattle and sheep may actually be due to the absorption of ammonium carbamate or some related nitrogen compound.

Summary. Administration of ammonium carbamate either orally or intravenously produced symptoms in ruminating lambs similar to those observed during acute urea toxicity. It is proposed that urea toxicity in the ruminant is not due to ammonia, but to ammonium carbamate. It is suggested that conditions exist in the rumen after the oral ad-

ministration of urea which would lead to the formation of ammonium carbamate.

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Alterations of Pancreas During Cortisone Diabetes in Rabbits.* (21731)

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Clinical diabetes due to excessive adrenocortical function or over-dosage with cortisone or ACTH is a well recognized phenomenon (1-3). Experimentally, a temporary steroid diabetes was first produced in rats by compound B and F and corticotropin(4). This type of diabetes characteristically showed, on a constant dosage, a gradual development of glycosuria which then gradually declined to become reestablished on increasing the dosage. This adaptation of the intact animal to the administration of the diabetogenic agent is classically also observed in the diabetes produced in dogs by anterior pituitary extracts (APE)(5,6). The morphologic sequence of events in the pancreas which might account for this phenomenon of adaptation has been worked out for hypophyseal but not for steroid diabetes. It is considered that the initial response of the pancreas to APE is an increased functional activity of the beta cells as evidenced by degranulation and hyperplasia. This is followed by hydropic change, which, if allowed to persist, ultimately results in beta cell destruction and a permanent diabetes(7, 8). As recently as 1950, it was stated that cortisone causes no observable pathologic changes in the pancreas of the rat(9). However, in the same year, severe diabetes and hydropic change of the islets after large amounts of cortisone were reported in a single rabbit(10) and subsequently confirmed(11). Prolonged diabetes has also been induced in guinea pigs by progressively increasing doses of cortisone(12). This was associated morphologically with hydropic change and islet hyperplasia. Furthermore, the pancreatic lesions persisted for some weeks after withdrawal of steroid therapy, despite remission of the glycosuria. In a more recent report on cortisone diabetes in rabbits, resistance to the diabetogenic agent was noted, but neither hydropic degeneration nor islet hyperplasia was observed(13). On the other hand, small doses of cortisone have been shown to cause islet hyperplasia and prevent the development of diabetes in the partially depancreatized rat (14). Furthermore, extreme centroacinar and islet hypertrophy and hyperplasia have been reported in monkeys after cortisone(15).

This would seem to indicate that an adaptive mechanism in the pancreas might account for the resistance of intact animals to the diabetogenic action of cortisone. The present experiments were designed to study the morphologic changes in the rabbit pancreas in an attempt to elucidate this phenomenon.

Materials and methods. Thirty-six rabbits of either sex, weighing between 2 and 5 kilos, were studied, of which 6 served as controls. Thirty were given cortisone acetate† in saline suspension, intramuscularly. The dosage var-

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† Cortisone acetate was supplied through the generosity of Dr. C. H. O'Donovan of the Upjohn Co.

ied from 12.5 mg to 75 mg daily for periods ranging from 3 days to 6 weeks. All animals were placed in individual metabolic cages and were allowed continuous free access to weighed amounts of Purina rabbit chow and water. Each rabbit was weighed bi-weekly. Twenty-four hour urine specimens were collected 3 times weekly in clean vessels to which toluol had been added. Urine glucose was determined by Benedict's quantitative method (16). Specimens from pancreas were fixed in Zenker formol and stained with the Masson trichrome, the Gomori chrome-hematoxylin, (17) and the aldehyde fuchsin methods(18). In addition, glycogen was demonstrated by the Periodic Acid Schiff(19), counterstained with iron hematoxylin, orange G-aniline blue (20), and controlled by diastase digestion.

Results. All animals showed some debility due to cortisone administration and many died within 10 days to 4 weeks after initiating therapy. The rabbits with the highest initial weight seemed to tolerate the cortisone best, and showed the most marked glycosuria. Treated animals showed an initial weight gain varying from 150 to 250 g which then declined to the starting value by 10 to 14 days. Those which survived longest showed a reduction in body weight which in many instances was 20 to 30% of the initial level. Weight loss could not be related directly to either changes in food intake or quantity of urinary glucose.

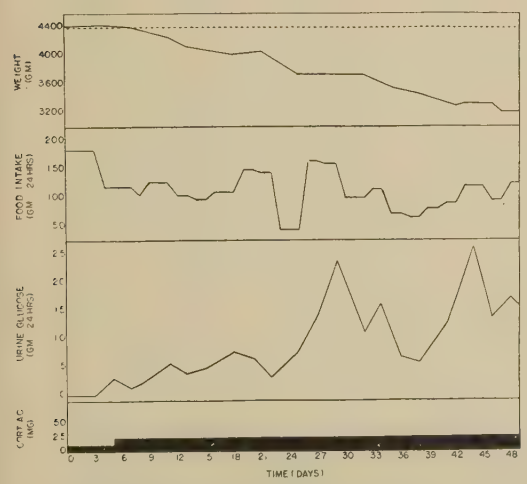

FIG. 1. Effect of cortisone administration on glucose excretion, food intake, and weight of a normal rabbit.

The rabbits differed markedly in the severity of the glycosuria that developed. In some, there were many days in which no urinary glucose was found. In others, it varied from a trace to 25 g daily (Fig. 1). Generally, there was a gradual appearance of a glycosuria between the 2nd and 5th day after starting treatment, that reached its maximum between the 5th and 9th days, and then gradually regressed. On increasing cortisone dosage, glycosuria was reestablished, reached a peak and subsequently declined. The greatest severity of diabetes appeared in animals with the lower cortisone dosages. Higher dosages seemed to diminish the diabetogenic action, but increased morbidity and mortality.

Morphologically, the most marked change consisted of a proliferation of the intercalated ducts, including the centroacinar cells, and changes in size and shape of the islets (Fig. 3). As seen in Fig. 3, there is extensive hyperplasia and hypertrophy of the intercalated ducts, making them much more prominent. In some instances, the prominence is accentuated by the presence of brilliantly stained material in the lumen. In addition, varying degrees of periductular fibrosis occasionally accompanied this hyperplasia. These changes were distributed throughout the pancreas, but were more marked in some areas. The interlobular ducts were not involved in this process and acinar dilatation was not found. The contour of islets in treated animals became irregular (Fig. 3), and they were often divided into lobules by strands of connective tissue. On closer examination, these "Mulberry" islets appeared to be formed by the continuity of portions of proliferating ductules with islet tissue and by intra-islet ductular proliferation (Fig. 5). In many cases, there was also intermingling of some acinar with the islet and duct tissue. The proliferative changes seemed to depend more on the length of therapy than on the degree of diabetes which developed, and was not observed prior to 2½ weeks after starting cortisone. In animals treated for 3½ weeks or longer it was uniformly present.

In addition, the well-known lesions of experimental diabetes, namely, degranulation and increased mitotic activity of the beta cells and glycogen infiltration (hydropic change)

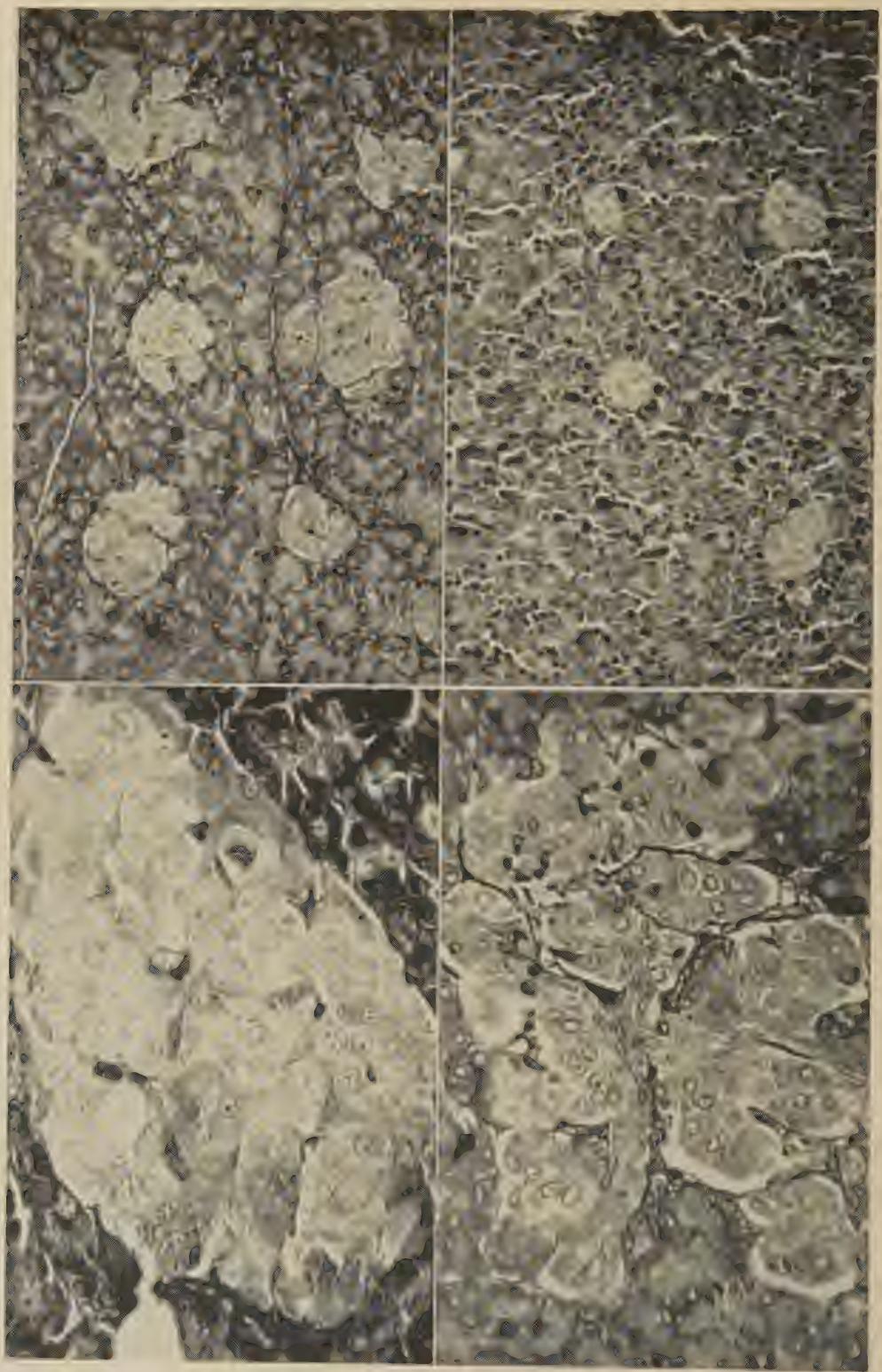

FIG. 2 (top left). Normal rabbit pancreas illustrating regular contour of islets and occasional barely visible intercalated ducts. PAS, trichrome × 125.

FIG. 3 (top right). Pancreas from rabbit treated with cortisone for 6 wk, illustrating irregularity in contour of islets and marked proliferation of ductular tissue. PAS, trichrome × 125.

FIG. 4 (bottom left). Islet from rabbit after 6 days of cortisone showing a mitotic figure in a beta cell, degranulation of the beta cells and a hydropic cell. PAS, trichrome × 370.

FIG. 5 (bottom right). A "Mulberry" islet from pancreas of a rabbit treated with cortisone for 5 wk illustrating lobulated appearance with ductular tissue entering into and intermingling with islet tissue. In addition, some hydropic beta cells are present. Masson trichrome × 200.

of ductular epithelium and of beta cells were observed. The beta cell degranulation was most prominent at early stages. At that time, numerous mitotic figures were also frequently observed in the beta cells (Fig. 4). These two phenomena occurred within 3 days after instituting therapy and were present up to 10 days, regardless of the degree of diabetes which developed. Thereafter, beta cell degranulation was present, but less uniformly. Similarly, glycogen infiltration of the small ductules and centroacinar cells was a frequent finding in animals with only very mild and short-lived glycosuria. Glycogen infiltration in the beta cells seemed to require a greater degree of diabetes of longer duration. However, occasional hydropic cells could be observed in most animals regardless of the severity of the diabetes. It is interesting that hydropic change was not observed in actively proliferating ductular tissue. In many islets there was marked glycogen infiltration of a portion of the beta cells while others appeared normal.

Discussion. In some respects, the morphologic picture in the pancreas seems to go hand in hand with the metabolic picture. The early degranulation and increased mitotic activity of the beta cells would seem to represent increased functional activity and may account for the early increasing resistance to the diabetogenic action of cortisone. The glycogen infiltration of the beta cells, which is similar to that seen in all forms of experimental diabetes (11), has previously been assumed to result from exhaustion of the beta cells due to increased functional activity(21). An alternative view that it is a concomitant of the hyperglycemia *per se* has also been promulgated (22). This latter view obtains support from the fact that hydropic change of ductular cells, to which no increased functional activity is attributed, is as prominent as hydropic change of the beta cells.

The proliferation of the intercalated ducts, including the centroacinar cells, and the changes in the islets might also be thought of as a response to metabolic stress. New islet tissue formation from the proliferating ducts would then explain both the marked tolerance to cortisone which may develop, and the failure to produce permanent diabetes. On the other hand, it may be that hydropic change in the ductules, which is one of the earliest and most constant findings, results in ductular obstruction and the proliferation is merely a result of this obstruction. This latter hypothesis seems to be invalidated by the fact that the ductular proliferation does not occur in other forms of experimental diabetes, despite hydropic change of ductular epithelium. Similarly, this lesion cannot be attributed to hyperglycemia *per se* as it does not appear in other forms of diabetes.

In addition, it has been shown that general debility, uremia(23), and vit. A or B deficiency(24,25) may cause pancreatic lesions which in certain respects resemble those described after cortisone. They all differ, however, in that acinar dilatation is prominent, whereas after cortisone, it is not seen. Furthermore, in unpublished observations, chronically malnourished rabbits did not show the lesion whereas in rabbits treated with vit. A, D, and B complex, in addition to cortisone, similar proliferative changes were found. Since cortisone interferes with the normal mechanism for resistance to infection, and since some of our animals died of Pasteurella pneumonia, it might be thought that the lesion here described was a form of pancreatitis. However, in animals treated with antibiotics and showing no evidence of infection, the lesion was equally prominent.

The proliferative changes cannot, therefore, in the light of present knowledge, be considered as merely the result of hyperglycemia or of some side action of the cortisone. It must, therefore, be postulated that cortisone, like certain other steroids(26), exerts a stimulative effect on the growth of the ductular tissue of the pancreas and probably in this way induces new formation of islet tissue. This might account for the adaptation of the rabbit to the diabetogenic action of the cortisone. However, the markedly diminished sensitivity of steroid treated animals to the action of exogenous insulin(27), leaves this in doubt.

Summary. 1. Cortisone acetate administered intramuscularly to rabbits caused the gradual appearance of glycosuria which reached a maximum at the 5th to 9th days and then declined. Increased dosage reestablished the glycosuria but it again regressed. Morphologically, the most prominent lesion observed was ductular and centroacinar proliferation and the formation of irregularly shaped and "Mulberry" islets. In addition, numerous mitotic beta cells were observed up to 10 days after starting cortisone treatment.

Similarly, degranulation of the beta cells was most prominent at early stages. Glycogen infiltration seemed to appear first in the ductular tissue and required a greater degree and duration of diabetes before affecting the beta cells. 2. The degranulation and increased mitotic activity of the beta cells are thought to be a response to hyperglycemia as is the glycogen infiltration of ducts and beta cells. The proliferative activity on the other hand is considered to represent a more direct action of cortisone on the pancreas.

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Relative Sensitivity of Circulating Eosinophils and Capillary Resistance to Exogenous Cortisone.* (21732)

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(With the technical assistance of G. B. Green.)

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The work of Kramár and associates(1-6) has shown that the hormones of the pituitary-adrenal cortical system exert a major controlling influence on the resistance of subcutaneous capillaries to rupture by external suction. These studies showed that both ACTH and cortisone increased the capillary resistance in the intact albino rat and dog, while adrenalectomy caused a permanent decrease to low levels in both species. In adrenalectomized animals cortisone but not ACTH elevated the resistance. Kramár(6) also found that pituitary growth hormone lowered capillary resistance below the normal level and that this effect was antagonized by cortisone. These workers believe that the actual level of the capillary resistance is largely determined by the relative amounts of these 2 hormones present. In the albino rat, physical and emotional stresses that are known to activate the pituitary-adrenal cortical system caused a characteristic response of the capillary resistance and there was an inverse relationship between the capillary resistance and the level of the circulating eosinophils(2,3,5). Wilhelmj(7) and Kramár (8) studied the changes in capillary resistance and the level of the circulating eosinophils during fasting and realimentation with diets high in carbohydrate or protein. In the dog, it was found that during prolonged fasting there was a progressive and maintained elevation of capillary resistance and a decrease in circulating eosinophils and that these changes were antagonized by exogenous pituitary growth hormone(9). On the basis of the accumulated evidence, the latter investigators concluded that the resistance of the capillaries and the level of the circulating eosinophils could be used as joint indices of the approximate degree of activity of the pituitary-adrenal cortical system.

During the course of their studies, Wilhelmj and co-workers noted that the temporal and quantitative relationships between the changes in capillary resistance and the eosinophil level often suggested that the eosinophil level reacted to lower concentrations of endogenous cortisone than did the capillary resistance.

The purpose of the present studies was 2-fold: *First,* to determine in the intact normal dog the threshold doses of exogenous cortisone necessary to produce significant changes in capillary resistance and eosinophil level; *second,* to investigate the possible effect of qualitative and quantitative changes in diet on these doses. The intact rather than the adrenalectomized dog was used because of the possibility of changes in threshold for the cortical hormones in the latter preparation and also because it was desired that the quantitative results be applicable to the intact animal.

Methods. Eight trained, standardized dogs, 6 females and 2 males were used. In order to avoid the emotional tensions often engendered by an unfamiliar investigator(10), all experiments were done by one person (D.S.). Total white cell and eosinophil counts were done in a Spencer bright line hemocytometer and the diluting fluid of Manners(11) was used for the eosinophil counts. Capillary resistance was done on the skin of the anterior abdominal wall by the method described by Kramár and Simay-Kramár(1). Great care was taken to avoid or to recognize capillary spasm which may cause a false elevation of the resistance value. Schering's cortisone acetate was used and the total daily dose was given in one intramuscular injection just before the single daily feeding. Hematologic and capillary resistance studies were done 20 to 22 hours later. Cortisone was given in variable amounts and

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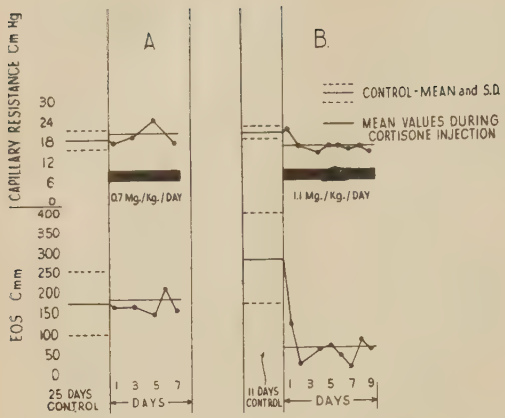

FIG. 1. Effect of varying amounts of exogenous cortisone on capillary resistance and circulating eosinophils in dogs on normal kennel diet.

each quantity was given for several days or until the observed changes became fairly stable. Following each period of cortisone injection a recovery period was given to allow the values to return to normal and avoid excessive inhibition of the pituitary-adrenal cortical system.

During the control and normal diet periods the animals were fed a weighed quantity of Nutrena dog food in an amount which maintained constant body weight at an optimal level. Diets high in carbohydrate (cracker meal) or in animal fat (beef suet and butter with cracker meal) were flavored and moistened with a solution of Difco Beef Extract and fed at the luxus consumption level of intake (120 cal/M^2/hr/24 hr). The high fat diets contained 50% or more of the calories from fat. Ground horse meat was used as the high protein diet and fed at the same caloric level.

Results.† (A) *On the normal kennel diet.* Thirteen experiments were done on 4 dogs with following results: 1) Cortisone in amounts less than 1 mg/kg/day caused no significant or consistent changes in either the level of the circulating eosinophils or the capillary resistance in one experiment on each of 2 dogs (Fig. 1A). 2) Amounts ranging from 1.0 to 1.9 mg/kg/day caused a significant fall in the circulating eosinophils but no significant elevation of capillary resistance in 5 out of 6 experiments on 4 dogs (Fig. 1B), Fig. 2A). 3) Amounts ranging from 2 to 2.8 mg/kg/day caused a significant elevation of capillary resistance as well as a significant fall in eosinophils in 4 out of 5 experiments on 4 dogs (Fig. 2B). Fig. 2B demonstrates the importance of using both capillary resistance and eosinophil level as joint indices of the approximate level of pituitary-adrenal cortical activity since when the eosinophil level has been maximally depressed by the minimal amount of cortisone, excess cortisone can cause no further eosinopenia but the elevation of the value of the capillary resistance detects the additional quantity.

(B) *Luxus consumption diets high in carbohydrate or animal fat.* When either of these diets were given to normal dogs without a preliminary fast they gradually altered the value of the capillary resistance and the eosinophil count so that by the time nutritional equilibrium had been reached both values were below normal. In 6 experiments on 3 dogs receiving the high carbohydrate diet, the minimal amount of cortisone necessary to cause a significant elevation of capillary resistance was 2.5 mg/kg/day for 2 dogs and 3.0 mg/kg/day for one dog. Thus, on this diet the minimal amounts of cortisone required overlapped the

† Significance was determined by t, using the small sample formula. The 0.05 level of confidence was considered as significant.

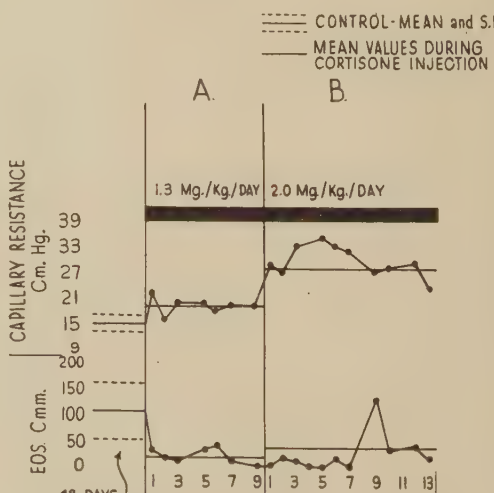

FIG. 2. Effect of varying amounts of exogenous cortisone on capillary resistance and circulating eosinophils in dogs on normal kennel diet.

highest value required on the kennel diet. In 6 experiments on 4 dogs on the high fat diet the amount of cortisone required to cause a significant elevation of capillary resistance ranged from 3.0 to 3.5 mg/kg/day. Since both diets caused the eosinophil level to approach the minimal level obtained by the threshold dose of cortisone, it was not possible to determine the relative sensitivity of capillary resistance and eosinophil level to exogenous cortisone on these diets.

(C) *Luxus consumption diets high in protein (horse meat).* On this diet the values for the capillary resistance and the eosinophil level were not changed from the normal. In 3 experiments on 3 dogs the amount of exogenous cortisone necessary to cause significant changes in both was the same as when the animals were receiving the standard kennel diet.

Summary. 1. When animals were receiving an optimal non-stress diet, exogenous cortisone in amounts less than 1 mg/kg/day caused no change in either the value of capillary resistance or the level of circulating eosinophils. Cortisone in amounts of 1 mg/kg/day or more caused a significant lowering of the eosinophil level while amounts of 2 mg/kg/day or more were required to cause a significant elevation of capillary resistance. 2. When the animals were fed luxus consumption diets high in animal fat, larger amounts of cortisone were required to produce a significant elevation of capillary resistance. 3. When the animals were fed a luxus consumption diet high in protein (horse meat) the quantitative responses to cortisone were the same as when they were being fed the optimal non-stress kennel diet of Nutrena. 4. Cortisone caused no consistent pattern of behavior of the total leucocyte count.

Conclusions. 1. The value of the capillary resistance and the level of the circulating eosinophils may serve as joint indices for the approximate degree of activity of the pituitary-adrenal cortical system. 2. On a non-stress diet (Nutrena dog food), the eosinophil level is about twice as sensitive to elevations in the cortisone level as is the capillary resistance. 3. The composition of the diet may alter these quantitative responses to cortisone.

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Preparation of Colloidal Fat Suspensions for Intravenous Use.* (21733)

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(Introduced by F. Glenn.)

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Colloidal suspensions of purified animal and vegetable fats have been prepared in our laboratory and those of animal origin have been given, without reaction, intravenously to animals and man. All previously reported preparations of fat for parenteral use have been prepared by high pressure homogenization in the form of emulsions stabilized by various emulsifying agents(1-4). Numerous modifications of fats and of emulsifying agents have been investigated but the preparation of fat in colloidal form has not heretofore been attempted.

Method. Colloidal suspensions of fat in water may be prepared in the following manner: One part of fat is dissolved in 10 parts of absolute alcohol at 80°C. Pyrogen-free water, maintained at 80°C, is then added very slowly with constant agitation until the total concentration of alcohol is about 50%. The alcohol is then evaporated on a water bath leaving the fat suspended in water. Further evaporation of the water may be carried out to any desired concentration and particles removed by filtration. From this stock suspension appropriate dilutions can then be made without disturbing its physical properties. Dextrose or any salt in isotonic concentration may be added at this point. The colloidal suspension may be sterilized by autoclaving in the container from which it is to be administered.

Results. The resulting suspension has a particle size of less than 250 mμ. It is therefore not visible under the oil immersion of the ordinary microscope (970x). It can be passed through a filter unchanged and exhibits rapid Brownian movement in the dark field microscope. It therefore satisfies the definition of a colloid in that the dispersion of the phase is of such a degree that the surface forces become an important factor in determining its properties. The stability of the fat preparation is thereby established. It can be autoclaved and stored indefinitely and does not break on the addition of salts in isotonic concentrations.

Discussion. The colloidal form of fat would seem to be ideal for intravenous use. No emulsifier is necessary, thus obviating the many toxic reactions due to the stabilizing agents. The particle size is so reduced that absorptive surfaces are greatly increased. In addition, the colloidal form of fat can be reconstituted in any isotonic medium. This removes one of the main hazards to the routine use of fat in emulsified form which breaks on the addition of salts. In this colloidal suspension the electrolytes, necessary for the maintenance of the severely ill patient, may be administered, simultaneously with the fat.

Reactions due to trapping of larger particles in the lungs and smaller capillaries of the body are also eliminated by the use of fat in colloidal form. Preliminary observations have been made in a group of patients who received 15% colloidal animal fat intravenously, in amounts up to 500 cc. Not a single reaction of even minor degree was observed. This is in contrast to previous experience with intravenous fat preparations (5-7). The results of the clinical studies will be discussed in a subsequent report.

The fate of the whole fat, injected intravenously without contamination from phospholipid emulsifiers, can also be investigated more easily. It is possible that the body cells may be able to utilize the colloidal form of fat more directly than the emulsified form.

Summary. A new method has been presented for the preparation of fat for intravenous administration. Fat prepared in this man-

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ner is in the form of a colloidal aqueous suspension with a particle size less than 250 mμ.

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Ribonuclease and Ribonuclease-Inhibitors Among Higher Plants. (21734)

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New inhibitors of ribonuclease should prove useful in the preparation of ribonucleic acids in native form, and in studies of the functions of ribonucleases as well. We have therefore looked for the presence of inhibitors among natural sources, and particularly among a series of common plants. Several species were found themselves to contain ribonuclease, and several others were observed to contain inhibitors.

Methods and materials. Preparation of extracts. Extracts were made from the following series of plants* which were collected in the vicinity of Cold Spring Harbor, L. I., during the months of July and August. Where different parts of the same plant were extracted separately, as leaves and flowers, the parts used are set off by parentheses: *Achillea millefolium* (flowers) (leaves); *Ailanthus altissima* (seeds) (leaves); *Allium* sp. (flower and seeds) (root); *Arisaema triphyllum* (berries) (root); *Asclepias syriaca* (flowers) (leaves); *Catalpa* sp. (flowers) (bean); *Cichorium intybus* (flowers); *Cornus florida* (leaves); *Daucus carota* (flowers); *Diervilla lonicera* (berries); *Fraxinus* sp. (leaves); *Hemerocallis fulva* (flowers); *Hibiscus syriacus* (buds and flowers); *Hydrangea quercifolia* (flowers); *Impatiens biflora* (flowers) (leaves); *Lathyrus* sp. (flowers and leaves); *Leontodon autumnalis* (flowers); *Linaria vulgaris* (flowers); *Lindera benzoin* (berries); *Liriodendron tulipifera* (leaves); *Lobelia cardinalis* (flowers, stem and leaves); *Lonicera japonica* (flowers); *Mentha spicata* (stem and leaves); *Mimulus ringens* (flowers); *Monotropa uniflora* (whole plant); *Myrica carolinensis* (leaves); *Phlox* sp. (flowers); *Phytolacca americana* (flowers and berries); *Rudbeckia hirta* (flowers, stem and leaves); *Salix nigra* (leaves); *Sassafras albidum* (leaves); *Silene noctiflora* (flowers and leaves); *Smilacina racemosa* (berries and leaves); *Solanum dulcamara* (flowers and leaves); *Symplocarpus foetidus* (stems); *Syringa vulgaris* (leaves); *Tilia* sp. (fruit); *Typha angustifolia* (pistillate flower); *Viburnum opulus* (berries); *Wisteria frutescens* (flowers) and *Yucca filamentosa* (flowers). Approximately 10 g of fresh plant tissue were minced with scissors and placed with 1 to 5 times this weight of demineralized water in a Waring Blendor equipped with a semi-micro monel metal container. After blending 1 to 6 minutes, considerable heating occurring in the longer runs, the homogenates were spun in an angle centrifuge for 10 minutes at approximately 10,000 G. The sediments were discarded and the supernates were stored at −20° prior to testing them. If a precipitate was present after thawing, the extract was re-

* The botanical terminology is that of Gleason(1).

centrifuged in order to provide a clear solution. *Measurement of ribonuclease activity.* The method used was a turbidimetric one similar to those described by McCarty(2) and Lamanna and Mallette(3). The course of depolymerization of ribonucleic acid was followed by withdrawing at appropriate times 1 ml samples of reaction mixture, adding them to 1 ml N HCl, and measuring the turbidity in a Beckman DU spectrophotometer at 420 mμ. *Survey of plant extracts.* (A) The plant extracts were examined for capacity to inhibit pancreatic ribonuclease by mixing in the following order a given amount of extract, 0.5 μg crystalline trypsin-free pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) contained in 0.5 ml 0.1% gelatin, 1 ml 0.1% gelatin, and sufficient 0.025 M veronal buffer, pH 7.2, to bring the volume to 7.0 ml. After 5 minutes at 37°, 3 ml H_2O containing 6.66 mg sodium ribonucleate (Schwarz Laboratories, Inc., Mt. Vernon, N. Y.) were added. Samples were withdrawn at 10 minute intervals for 40 or more minutes. The quantities of plant extract used were 1, 0.1, 0.01 ml and none. (B) The extracts were tested for ribonuclease activity by omitting the pancreatic ribonuclease from the system described under (A).

In later measurements of ribonucleases and their inhibition two modifications of the method described were made: (1) Since the precipitates obtained on addition of samples of reaction mixture to HCl were found on standing to slowly undergo solution, the interval of time between adding the sample to HCl and reading the turbidity was fixed at 30 minutes. (2) When the effects of inhibitors were studied, the mixtures containing ribonuclease and solutions of inhibitor were allowed to interact for 20 minutes at 37° before addition of sodium ribonucleate.

Results. Of a total of 48 extracts, 6 were able themselves to degrade ribonucleic acid, 3 gave evidence of inhibiting pancreatic ribonuclease, and 39 showed no clear-cut evidence either of possessing ribonuclease activity or of inhibiting the action of pancreatic ribonuclease.

The extracts which degraded ribonucleic acid were those derived from *Phlox* sp. (flowers), *Lathyrus sp.* (leaves and flowers), *Asclepias syriaca* (leaves), *Asclepias syriaca* (flowers), *Allium* sp. (flowers and seeds) and *Yucca filamentosa* (flowers). The extracts of *Yucca* and *Allium* were considerably more active than those of *Phlox, Lathyrus* and *Asclepias.*

Extracts *inhibiting* the degradation of ribonucleic acid by pancreatic ribonuclease were those obtained from bayberry (*Myrica carolinensis*) leaves, dogwood (*Cornus florida*) leaves and lilac (*Syringa vulgaris*) leaves. The first two extracts were found to form insoluble complexes with gelatin alone, and they inhibited apparently by coprecipitating the gelatin and ribonuclease in the test system. They were not studied further. The lilac extract, however, precipitated neither gelatin nor ribonucleic acid, and it appeared to inhibit degradation of ribonucleic acid by reacting with the enzyme.

Inhibitor in lilac leaf extracts. Extracts that were somewhat more active than those obtained using the Waring Blendor could be prepared by grinding lilac leaves in a mortar with an equal weight of quartz sand and 2 volumes of water, followed by centrifugation of the expressed fluid. Active extracts from larger quantities of leaves than can conveniently be handled by grinding in a mortar could be prepared with the aid of a "Juicex" juice extractor (Drachenberg Products Mfg. Co., Detroit, Mich.) Freshly collected leaves uniformly yielded extracts that were markedly inhibitory but leaves that had been stored in the cold for several weeks yielded extracts that were only weakly inhibitory. The extracts themselves could be stored at refrigerator temperature for several months without apparent loss in inhibitory capacity. The *inhibition of pancreatic ribonuclease* by lilac leaf extract is illustrated in Fig. 1, which shows also the course of depolymerization of ribonucleic acid as a function of time of interaction between enzyme and inhibitor. It can be seen that appreciable inhibition of ribonuclease occurred only when sufficient time was allowed for the inhibitor to act. The extent of inhibition was found to depend also on the temperature at which interaction occurred, being least at 10°, greatest at 37°,

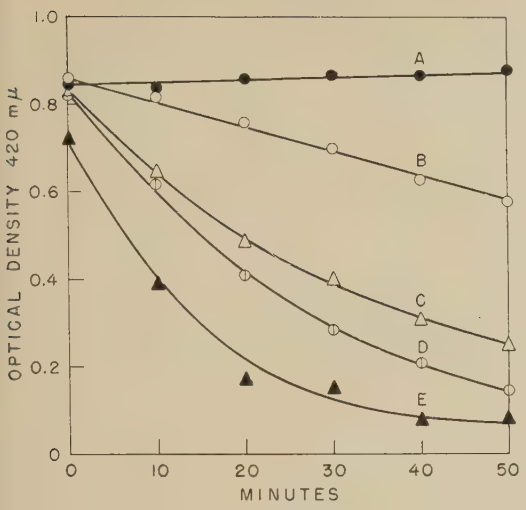

FIG. 1. Course of degradation of ribonucleic acid after allowing pancreatic ribonuclease to interact with inhibitor from lilac leaf at 37° for 20′ (Curve A), 10′ (Curve B), 5′ (Curve C), 2½′ (Curve D) and 0′ (Curve E). In each instance 0.1 ml crude leaf extract was used, the final volume of the reaction mixture being 10 ml.

and intermediate at intermediate temperatures. In subsequent experiments, inhibitor and enzyme were incubated at 37° for 20 minutes prior to addition of ribonucleic acid. The degree of inhibition of ribonucleic acid depolymerization was found to depend on the concentration of lilac leaf extract used, but the precise quantitation of inhibitory activity was not achieved, partly because the degree of inhibition is apparently not a simple function of inhibitor concentration. The inhibitor was not precipitated from lilac leaf extracts by addition of trichloracetic acid or lead acetate or by salting out with ammonium sulfate. On dialysis, most of the inhibitory activity passed through cellophane. Using extracts that had been partially purified by removal of impurities with Pb, followed by treatment with H_2S, the inhibitor was found to be stable on exposure to 0.1 N HCl at 100° for 15 minutes, and to 0.1 N NaOH at 100° for 15 minutes. The inhibitory activity was, however, nearly completely destroyed by ashing with HNO_3, and that which remained was attributable to magnesium and calcium. Partially purified preparations show an absorption maximum at 278 mμ, but it is not known whether this property is due to the inhibitor itself or to an impurity.

Inasmuch as privet (*Ligustrum vulgare*) is closely related botanically to lilac, extracts of privet leaves were prepared. These inhibited the degradation of ribonucleic acid in a manner similar to that found for lilac leaves.

Specificity of inhibitor in lilac and comparison with other inhibitors. A comparison was made of the capacity of the lilac inhibitor to prevent the depolymerization of sodium ribonucleate by the ribonucleases in extracts of the 5 plant species previously referred to, by *Neurospora* ribonuclease (contained in the filtrate of a culture of *Neurospora crassa* kindly supplied by Dr. Sigmund Suskind), by spleen ribonuclease (a partially purified preparation kindly provided by Dr. Leon Heppel) and by pancreatic ribonuclease. The ribonucleases of pancreas and *Asclepias* were markedly inhibited, that of *Phlox* was inhibited much less, and the remaining enzymes were not significantly affected. The *degradation of ribonucleic acid* by pancreatic ribonuclease is known to be inhibited by Mg++ (3) and by heparin(4,5), and it was of interest to compare these agents with the inhibitor in lilac. It was found that Mg++ was considerably less specific than the lilac agent since the former markedly reduced the activity of all ribonucleases except those from spleen and *Lathyrus*. Heparin, in the concentration tested, markedly reduced the activity only of pancreas and spleen enzymes, and gave a pattern of inhibition different from that seen with the inhibitor in lilac.

Summary. 1) Aqueous extracts prepared from 41 common plants were tested for ribonuclease activity and for capacity to inhibit pancreatic ribonuclease. Extracts of 5 plants (*Phlox* sp., *Lathyrus* sp., *Asclepias syriaca, Allium* sp. and *Yucca filamentosa*) were found capable of degrading ribonucleic acid, and these plants appear to constitute new sources of ribonuclease. It is known that similar enzymes are present in wheat(6), in sprouting soy beans(7) and in various other types of germinating seeds(8-10), but there appears to be little information concerning the occurrence and distribution of ribonuclease in the plant kingdom in general. 2) A new, naturally occurring inhibitor of pancreatic ribonu-

clease is shown to be present in lilac leaves. It is a thermostable dialyzable substance of unknown identity. An apparently similar substance is present also in privet leaves. The agent present in lilac inhibits not only pancreatic ribonuclease but also *Asclepias* ribonuclease, and to a less extent *Lathyrus* ribonuclease. The specificity of the lilac inhibitor is compared with that of magnesium ion and heparin.

The authors are indebted to Miss Lois Schwartz for technical assistance during the later phase of this work.

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Effect of Polyvinyl Pyrrolidone on Immune Response in Mice.* (21735)

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Previous studies(1) on the effects of administration of polyvinyl pyrrolidone (PVP) to mice demonstrated considerable reticulo-endothelial storage of this compound, or a derivative of it, in liver and spleen. Entry of PVP into reticulo-endothelial cells was accompanied by displacement of previously stored colloidal dyes, or by inhibition of such storage if dye was administered simultaneously with, or subsequent to, injection of PVP. In view of this influence of PVP on storage function of reticulo-endothelial tissues, it was of interest to investigate whether antibody production which presumably involves the macrophage system, was affected by PVP. Since work carried out in this laboratory during the past 6 years(2-6) had given ample information on the immune response of mice to sheep red cells, this particular approach was selected.

Material and methods. Three-to 6-month-old, healthy mice of strains C57BL, C3H, C57L, AKR and CBA were used. Two lots of PVP† were used: (1) Ga-PVP-140 with a K value of 33; (2) G-12306 with a K value of 84. PVP was used as 3.5% solution in physiologic saline, neutralized to pH 7 by addition of N/10 NaOH; fresh solutions were prepared weekly. Injections of PVP were given subcutaneously or intraperitoneally as indicated for the individual series of experiments later on. The dosage per injection was 0.5 ml of the 3.5% solution (17.5 mg per mouse). *Two different procedures* for immunization with sheep red cells were used: (a) one was identical with the method used in our previous work(3,6), consisting of 6 intraperitoneal injections each of 0.1 ml of a 0.1% suspension of sheep erythrocytes given 3 times weekly; (b) the alternative method consisted of a single intraperitoneal injection of 0.1 ml

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† The compounds were generously supplied by the General Aniline and Film Corp. through Dr. D. B. Witwer.

TABLE I. Series I: Immune Antibodies for Sheep Red Cells in PVP-Treated C57BL Mice.

Group	No. of animals	Titer of antibodies: Agglutinin 16	32	64	Mean	$\bar{x} \pm$ S. D.	P*
Control	15	3	9	3	32	2.8	.09
PVP	15	7	6	2	25	2.7	
		Hemolysin† 1280	2560	5120	10,240		
Control	15	4	3	5	3	3538	10.2	.04
PVP	15	6	7	1	1	2222	8.6	

* Indicates level of probability at which differences between control and PVP groups are significant when tested according to the method of Yates(8).

† Represents incipient hemolysis. Complete hemolysis was also determined and showed parallel trends.

of a 4% suspension. The first method will be referred to as multiple small dosages of antigen, the second as single large dose of antigen. In all instances animals were sacrificed by decapitation 7 days after the last (or single) injection of sheep red cells. The blood was collected, the serum used for determination of agglutinins and hemolysins (for technics, see previous communications(2,3)) and, in most instances, for determination of PVP according to the iodometric method devised by Cannan (7). Weights of spleen and liver were obtained in all animals; histologic sections of these tissues were examined, and PVP was estimated in liver and spleen in most experiments.

Results. Four series of experiments will be presented in detail which differed from each other in the number of PVP injections, the PVP compound used, the time relation between PVP administration and immunization and the method of immunization.

Series I: Animals of the experimental group received a total of 12 daily intraperitoneal injections of PVP (K 33), with 5 injections given per week; control animals received the same number of intraperitoneal injections of 0.85% saline. Three days after the last injection, animals of both groups were immunized with multiple small doses of antigen. On days between injections of sheep cells, mice of the experimental group received a total of 6 additional intraperitoneal injections of PVP, with control mice being injected simultaneously with saline. The results of antibody determinations in mice of strain C57BL are summarized in Table I. Similar results were obtained in an analogous experiment utilizing C3H mice. In both strains, PVP-treated animals showed lower antibody levels than the controls. Calculation of geometric means and standard deviations of the titers followed procedures detailed in recent reports (5,6). Tests of significance were done according to the method of Yates(8).

Series II: Mice of the experimental group received a total of either 12 or 18 daily subcutaneous injections of PVP (K 33), with 5 injections given weekly; controls received no injections at all. On the day after the last injections of PVP, immunization with multiple small doses of sheep cells was started in all groups. No further injections of PVP were given and the experiment was terminated seven days after the last injection of sheep red cells. Both groups of PVP-injected C3H mice showed slightly lower mean titers than the controls; these differences were not found statistically significant except in the case of complete hemolysis in strain C3H.

Series III: A group of 25 C3H mice was given a course of immunization with multiple small doses of antigen. During the interval between the last injection of sheep cells and the sacrifice of the animals 7 days later, 15 mice were given 6 subcutaneous injections of PVP (K 33) with the remaining 10 mice serving as controls. Antibody levels in control and experimental groups showed no difference worthy of note.

Series IV: In each experiment, 27 mice were divided into 3 equal groups receiving the following treatment: (a) none (controls); (b) 9 subcutaneous injections of PVP (K 33); (c) 9 subcutaneous injections of PVP

TABLE II. Series IV: Immune Antibodies for Sheep Red Cells in PVP-Treated Mice of 5 Inbred Strains.

Strain	Group	Agglutinin Mean	Agglutinin ± S. D.	Agglutinin P*	Hemolysin* Mean	Hemolysin* ± S. D.	Hemolysin* P*
C57BL	Control	49	4.5		104	28.6	
	PVP 33	16	4.0	.01	40	27.1	.19
	84	19	4.1		86	11.1	
C57L	Control	19	6.0		42	12.0	
	PVP 33	7	11.8	.72	10	19.2	.38
	84	22	9.0		19	21.5	
C3H	Control	28	7.6		127	8.3	
	PVP 33	12	8.3	.02	20	20.6	.001
	84	5	7.0		5	7.8	
CBA	Control	43	8.3		254	9.7	
	PVP 33	9	4.6	.50	3	13.5	.04
	84	47	10.3		34	13.9	
AKR	Control	87	8.1		17	17.2	
	PVP 33	37	4.4	.08	22	9.6	.03
	84	37	6.8		3	8.0	

* See footnotes to Table I.

(K 84). Mice of 5 different strains (C57BL, C57L, C3H, CBA, AKR) were treated in this manner. On the day following the last PVP injection all animals received an intraperitoneal injection of a single large dose of antigen. On succeeding days, groups (b) and (c) received 5 injections of the PVP solution used for them prior to immunization, and all animals were sacrificed on the 7th day after the sheep cell injection. Table II summarizes the mean antibody titers recorded for the 3 groups of each of the 5 strains. In strains C57BL and C57L, hemolysin titers in control and experimental groups did not differ significantly from each other. On the other hand, significantly lower hemolysin levels were present in PVP-treated mice of strains C3H, CBA and AKR. In C3H and AKR, but not in CBA mice, PVP 84 was more effective than PVP 33 in depressing the titer of hemolysin. Levels of agglutinins were affected as follows: significant lowering in mice given PVP occurred in strains C57BL, C3H, AKR, but not in C57L and CBA.

TABLE III. Series IV: Effect of PVP on Splenic Weights (mg).

Strain		Control	K 33	K 84
C57BL	Range	64-103	81-144	81-146
	Mean	79.7	105.3	103.3
	± S. D.	13.9	19.8	24.7
	Increment, %*	—	31	29
C57L	Range	75-119	84-118	94-127
	Mean	95.7	102.2	106.0
	± S. D.	15.2	11.4	10.7
	Increment, %*	—	6	10
C3H	Range	83-116	95-121	140-177
	Mean	101.0	110.0	160.8
	± S. D.	11.4	9.3	13.8
	Increment, %*	—	9	60
CBA	Range	83-178	123-216	155-300
	Mean	127.8	164.0	226.1
	± S. D.	39.8	29.0	45.6
	Increment, %*	—	36	98
AKR	Range	59-170	82-240	80-217
	Mean	78.4	121	129.4
	± S. D.	33.9	48.8	42.3
	Increment, %*	—	68	86

* As compared with value in control group.

Effects of PVP on weights of spleen and liver. Table III contains pertinent data on spleens of animals of series IV. Presence and degree of splenomegaly was most marked in mice of strains C3H, treated with PVP 33 or PVP 84. No increase of splenic weights was found in C57L mice, and increases observed in C57BL mice were less pronounced than those recorded for the strains mentioned previously. Weights of livers were not found to be changed to any conspicuous degree in mice of any of the strains, regardless of type and dosage of PVP administration.

Concentration of PVP in serum, liver and spleen. Values obtained in Series IV, recorded in Table IV, failed to show any consistent relationship between PVP levels, strain, and effect of PVP on antibody level.

Effect of PVP in Vitro on Agglutinin and

TABLE IV. Series IV: Concentration (%) of PVP in Serum, Liver and Spleen.

Strain		Serum		Liver		Spleen	
		K 33	K 84	K 33	K 84	K 33	K 84
C57BL	Range*	.33-.62 (9)	.69-1.31 (8)	.46-.59 (8)	.46- .85 (9)	.46-.69 (4)	.25-.99 (4)
	Mean	.47	.95	.54	.66	.59	.63
	± S. D.	.09	.23	.04	.13	.03	.30
C57L	Range*	.28-.45 (4)	.26- .44 (5)	.30-.53 (8)	.36- .61 (9)	.14-.69 (4)	.15-.40 (4)
	Mean	.36	.36	.41	.48	.41	.28
	± S. D.	.08	.08	.07	.09	.22	.12
C3H	Range*	.28-.51 (6)	.64-1.22 (6)	.29-.48 (9)	.54-1.38 (9)	.12-.49 (4)	.28-.54 (4)
	Mean	.36	.90	.39	.96	.29	.42
	± S. D.	.08	.20	.06	.10	.17	.11
AKR	Range*	.09-.42 (9)	.18- .47 (9)	.28-.49 (9)	.28- .40 (9)	.54-.84 (5)	.15-.75 (4)
	Mean	.25	.38	.36	.35	.72	.46
	± S. D.	.12	.17	.06	.04	.15	.25

* Figures in parentheses denote No. of individual animals, or of pooled samples tested.

Hemolysin Activity. Since serum PVP concentrations may approach 1% after prolonged administration of PVP, the *in vitro* effect of this compound on hemagglutinins and hemolysins was tested. Final concentrations of PVP (K 33 or K 84) of 0.1, 0.5, 1.0 or 1.25% had no noticeable effect on the hemolytic titer of antisheep erythrocyte rabbit and anti-sheep erythrocyte mouse serums; a final concentration of 2.5% of PVP depressed the hemolytic titer by one or 2 tubes. Hemagglutination was not affected by any of these concentrations.

Histologic Findings: Storage in reticuloendothelial cells was found in all tissues examined, with the greatest amounts present in spleen and liver. The findings were comparable to those described previously(1).

Discussion. The fact that administration of PVP in some of the experiments described caused a depression of levels of immune hemoantibodies as compared with untreated controls may not be surprising. It seems to be more significant that this phenomenon occurred only under certain conditions which can be defined to a certain extent by comparing the different experimental series:

1. *Strain differences.* In series IV, 5 different strains were compared and inhibition of immune response as judged from the titer of hemolysin for sheep red cells was present in 3: C3H, CBA and AKR, but not in C57BL or C57L. These findings are of special interest in connection with previous work by Davidsohn and Stern(6) according to which immune response to administration of hemoantigens regularly results in high levels of antibodies in strains C57BL and C57L, whereas mice of strains C3H, CBA and AKR are inferior in their ability to produce these antibodies.

2. *Differences in procedure of immunization.* Depression of levels of sheep hemolysin by PVP was more marked in series IV in which the single large dose of antigen was given as compared with series I in which multiple small doses were used. This finding is in good accord with observations made by us (9) in connection with the effect of presence of tumors on levels of immune hemoantibodies: impairment of production of hemoantibodies became apparent in tumor-bearing animals only when single large doses of antigen were given, but not when multiple small doses were employed. Possibly immunization with single large doses of antigen places a more severe strain on the antibody-producing tissues and hence this is a more sensitive test for detection of deficient antibody formation. One also might connect this finding with the studies of Taliaferro and Taliaferro(10) who demonstrated that the spleen is primarily involved in antibody production after administering single large doses of hemoantigen whereas nonsplenic sites of antibody production are more active after administration of multiple small doses. On the other hand, there seemed to be no relationship between hemoantibodies and concentration of PVP in serum, liver, or spleen (Table IV).

3. *Timing of PVP administration.* According to series III it is necessary to administer PVP prior to administration of the hemoantigen, in order to influence antibody production. On the other hand, series II showed that the depressing effect of PVP was less pronounced if it was given only prior to hemoantigen, but not afterwards as was done in series I and IV. Hence it is conceivable that the interference of PVP with antibody production may be connected with the role of the macrophage system in phagocytosis of the particulate red cell antigen and preparing in some way its utilization for antibody formation. Failure to administer PVP after administration of the antigen may favor regeneration of reticulo-endothelial cells, compensating for the functional impairment of macrophages caused by the preceding administration of PVP.

4. *Molecular size of PVP.* Series IV as well as some other data, not tabulated in detail, showed that PVP with predominantly larger molecular size (K 84) depressed hemoantibodies more effectively than K 33, the PVP compound of lower molecular size. Most likely this phenomenon is related to the ease with which the compounds can be excreted, which is known to be inversely proportional to molecular size.

5. *Dosage of PVP.* Review of the experimental series described in detail as well as of other experiments not tabulated showed that a minimum of 6 injections of 0.5 ml of the 3.5% solution of PVP was required for depressing the immune response. In most series, 12, 14, or 18 injections were given. In series II no difference in antibody levels was found between mice receiving totals of 12 or 18 injections, respectively. Roughly, administration of 0.5 ml of the 3.5% solution of PVP corresponds to infusion of 1 liter to man.

The experiments on the effect *in vitro* of PVP on hemolysin and agglutinin rule out the possibility that the lower antibody levels observed in PVP-treated mice are merely the result of the presence of PVP in the serum. Such an assumption was *a priori* unlikely on the basis of data obtained in series II and IV according to which fairly high serum PVP concentrations were not associated with depressed levels of hemoantibodies.

A few observations have been recorded recently regarding the possible effects of PVP on immune phenomena. Fresen and Weese (11) produced anaphylactic shock in rabbits sensitized with human serum, in spite of preceding administration of large doses of high-molecular "Kollidon" (PVP). Pautrizel and Lopes(12) found either augmentation or inhibition of cutaneous allergic reactions in presence of PVP, depending on concentration of PVP and timing of its administration. Dieckhoff(13) injected rabbits with PVP preparations of molecular weights of 40,000 or 80,000; subsequent immunization with *S. typhosa, Cl. tetani,* and *C. diphtheriae* did not show significantly different antibody levels in PVP-treated rabbits and controls.

The results of our own study appear to us of significance on 3 counts: (1) a new biologic property of PVP, a proposed plasma expander, was demonstrated in its ability to depress levels of immune hemoantibodies in the mouse under certain conditions; (2) the depressed immune response appeared to be correlated with the splenomegaly produced by PVP; (3) significant interference with formation of immune hemoantibodies was observed only in mice belonging to strains which have been found to be poor producers of such antibodies(6). This may indicate the need for selecting specific constitutional types of animals when it is desired to investigate the possible interference of a compound with the immune response. In other words, failure to find such an interference in healthy animals with especially well developed immune response should not be taken as proof that this will be also the case in animals with a different constitutional background, or in patients with severe organic or functional damage to their antibody-producing tissues.

Summary. (1) Administration of PVP to mice was found capable of depressing levels of immune hemolysin for sheep red cells. (2) This effect varied with the strain of mice used, timing of injection of PVP, type of antigenic stimulation and molecular size of the PVP compound. (3) Depression of hemolysin appeared to be correlated with the degree of

splenomegaly, but not with the PVP concentration in serum, liver and spleen.

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Antigenic Crossings within Poliovirus Types.* (21736)

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In vivo and *in vitro* neutralization tests have shown that the polioviruses† fall into 3 antigenic types, with no apparent crossing between strains belonging to different types (1,2). It is now customary to type strains by tissue culture (TC) neutralization tests in which an amount of virus, varying from 100 to 100,000 TC doses, is tested against a large excess of specific, immune serum. Strains may also be typed by their reaction in the complement-fixation (CF) test against standard antisera(3).

This report deals with a comparison of the two methods of typing in which 70 strains, shown to be monotypic in the conventional TC neutralization test referred to above, were typed by their complement-fixation reactions against 3 type specific sera. An attempt was made to determine whether the CF test might reveal crossings between strains belonging to different types. One important reason for carrying out this study was the number of hints that epidemiologically there might be some crossing or protection against Type 1 and 3 strains in populations having Type 2 antibodies(4-6).

Materials and methods. Standard TC passage strains included Brunhilde and Mahoney (Type 1), Y-SK and MEF1 (Type 2), and Leon and Saukett (Type 3). The CF reactions of these 6 passage strains were compared with those of 64 strains in early TC passages. The new strains were recovered chiefly from patients in the New England area, during the summer of 1954. Twelve of the strains were isolated by Dr. Dorothy M. Horstmann from young children in Egypt in 1952 and were kindly made available by her. Each strain was cultivated to high titer (over 10^{-6}) in monkey kidney monolayer cultures(7) grown in a medium consisting of 0.5% lactalbumin hydrolysate, 2.0% calf serum, and 97.5% Hanks' solution(8). All strains were tested as undiluted TC fluid against 3 standardized sera prepared in monkeys by repeated intramuscular inoculations with Brunhilde, Y-SK, and Leon TC grown viruses. The method of typing strains by neutralization tests in monkey kidney cultures as employed here has recently been reviewed(9). The plate CF test as used in this laboratory for poliovirus has also been described(10). Results are

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† The term *poliovirus* is used in accordance with the recommendation of the Virus Subcommittee of the International Nomenclature Committee of the International Congress of Microbiology(14).

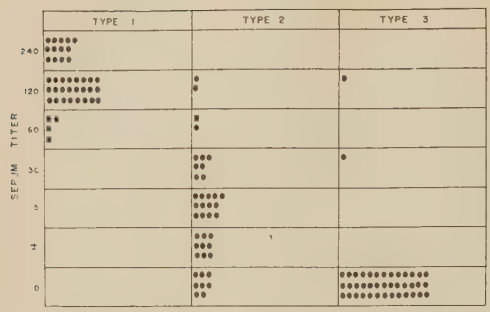

FIG. 1. CF crossings of Type 1 strains homotypic by neutralization tests. Serum titers of 3 standardized typing sera are shown.

expressed as serum titers or serum avidity scores against 4 to 8 units of each antigen. In the tests the amount of complement fixed at each dilution of antigen and antibody was measured, and the greater the amount fixed, the higher the avidity. The total avidity score is given in the results, and this is the sum of the avidity scores of all serum dilutions which fix complement(10).

Results. Standard TC passage strains. Against 4 to 8 units of antigen (Brunhilde, Y-SK, or Leon) which had been used for immunization, each standard serum yielded titers of 120 to 240. However, against the antigens of the two heterologous types the sera were negative at a dilution of 1 to 15. When the sera were tested against the Mahoney, MEF1, and Saukett antigens, the results were similar in that only homotypic reactions were observed.

Freshly isolated strains. Polioviruses isolated in 1952 and 1954, and limited to a few passages in TC, have all been monotypic in the TC neutralization test, but may be ditypic or, in rare instances, even tritypic in the CF reaction. Fig. 1 shows the CF results for 41 strains which were neutralized only by Type 1 serum. Each strain was used as antigen and tested against each of the 3 standardized sera. Thus each strain is entered in the Type 1, Type 2, and Type 3 columns. All 41 strains reacted with Type 1 serum at serum dilutions ranging from 60 to 240. A number of strains (42%) failed to react with the other sera. However, 54% of the strains reacted with Type 2 antibody, the serum titering from 15 to 120. Only 5% of the strains crossed with Types 2 and 3 sera. Fig. 2 shows similar crossings of the same 41 strains as determined by avidity scores: all reacted with Type 1 sera, 24 with Type 2, and 2 with Types 2 and 3.

The crossings of the strains identified as Type 3 by neutralization tests are shown in Fig. 3. This figure is almost a mirror image of Fig. 2 which presented the Type 1 results on a similar scale. Of the 19 strains tested, 6, or 32%, were monotypic; 11, or 58%, were ditypic; and 2, or 11%, were tritypic in the CF test.

Only 4 strains identified as Type 2 by neutralization tests were available for CF testing. All reacted with Type 2 serum, 2 strains crossed with Type 1 and one with Type 3 serum.

Discussion. The present findings may help to explain the heterotypic antibody responses which accompany, to varying degrees, the homotypic ones in poliomyelitic infections of man. When poliomyelitic infection occurs in man, the antibody response is not always limited to the type causing the disease(11,12). The heterotypic responses are much more pronounced in the CF, than in the neutralization, reactions. They are also much more pronounced in adults than in children, suggesting

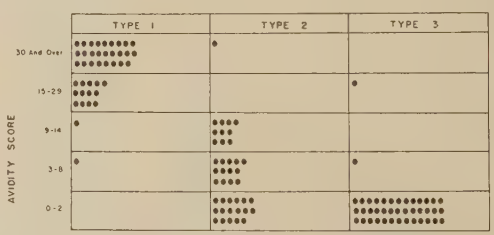

FIG. 2. Crossing of Type 1 strains according to CF avidity.

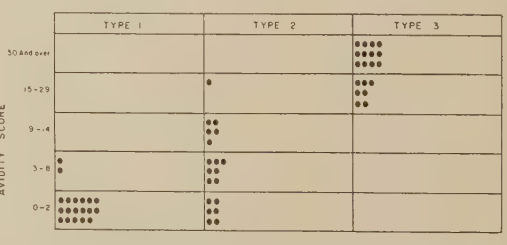

FIG. 3. CF crossings of Type 3 strains homotypic by neutralization tests. Avidity scores are shown for each antigen-serum titration.

that previous sensitization and the anamnestic response play a role. In any case, the broad serological response of poliomyelitis patients, and the epidemiological correlation between the presence of Type 2 antibodies and immunity to Type 1 or Type 3 strains in different populations, have suggested that polioviruses have group antigens in common. The present report offers direct evidence of antigenic overlap within poliovirus types. The available data suggest that the overlap between Type 1 and 2 strains, and again between Type 2 and 3 strains, is greater than between Type 1 and 3 strains. The designation of Lansing strains as Type 2, placing them between Types 1 and 3, was truly done with amazing foresight. In analyzing the neutralizing antibody responses of children vaccinated with formalinized vaccines, Salk(13) has come to a similar conclusion. He has found that children possessing Type 2 antibodies are sensitized so that they produce greater antibody responses to Type 1 and Type 3 antigens than do children lacking Type 2 antibodies; conversely, children possessing Type 1 or 3 antibodies behave as though sensitized to Type 2. In contrast, no such crossing was found between Type 1 and 3 antigens.

The present results of antigenic crossing differ from those obtained in the neutralization test. However, it is general practice in poliomyelitis laboratories to type new strains in the presence of a large excess of neutralizing antiserum. Whether the typings would be as sharp if minimal amounts of serum had been employed remains to be determined.

The six strains which had been through a number of serial transfers in TC gave only monotypic responses in the CF test. This raises the question as to whether recently isolated cross reactive strains will retain or lose this property on repeated passage.

Summary. Seventy poliovirus strains, identified as single types by their reactions in the neutralization test, were studied in the complement-fixation reaction. For each strain an antigen was prepared in monkey kidney culture and this was tested against three standardized sera, each representing a different type. About half of the newly isolated strains were found to be ditypic and showed Type 1 and 2, or Type 2 and 3, antigens. A few strains were tritypic. The major antigen identified in the complement-fixation test agreed with the type assigned to the strain from its reaction in the neutralization test. The findings may help to explain the heterotypic antibody response in poliomyelitis infection of man, and the epidemiological correlations between the prevalence of Type 2 antibodies and immunity to Type 1 or Type 3 strains.

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Regulation of Blood Flow in Cross-Circulation for Intracardiac Surgery.* (21737)

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Controlled cross-circulation is a promising technic in producing a dry field for intracardiac operations. By means of a single pump (Sigmamotor†) arterial blood from a donor perfuses the patients' aorta while the patients' venous return is passed to the donor (1-3). Thus, in the absence of venous inflow into the heart a relatively dry cardiac field is produced while adequate coronary and cerebral blood flow is maintained during the operating period. It is obvious that such a system must be so balanced that exactly the same quantity of blood flows from donor to patient as from patient to donor. One way flow can be easily determined, but it is the difficulty in exact balancing of 2-way flow that has heretofore greatly increased the danger of cross-circulation in cardiac surgery. During 30 minutes of cross-circulation 30 liters of blood may be exchanged in both directions. If the flow to the donor were reduced to 900 cc per minute while that to the patient remained at 1,000 cc the donor would have lost 3 liters in 30 minutes. This could be deleterious.

Thus, the detection and immediate correction of *differences* in bidirectional flow appear to be major problems which must be solved before cross-circulation can be applied with reasonable safety. It is the purpose of the present work to develop a simple practical method for the accurate regulation of the two-way flow under operating room conditions and to compare this method with others which are more complicated.

Method. In this study of the regulation of bidirectional flow, 2 methods for measurement were employed and compared: The first of these employs flow *rate* recorders, the other by simple weighing gives a *cumulative* record. In conjunction with the latter method, various modifications (2a,b,c,d) were tested. 1. *Flow rate* recorder: One 5734 vacuum tube bristle flowmeter(4,5) was inserted into the tubing leading from recipient to donor and another bristle flowmeter into the tubing from donor to recipient. Bristle flowmeters were chosen because of their great sensitivity (high signal to noise ratio), compactness and low tendency for fibrin deposition(6). The flow signal from each flowmeter was electrically integrated in order to record the mean rate of volume flow in each side of the circuit. The flow difference between the 2 sides was established with the aid of a "flow-difference-meter." This consisted of a galvanometer suitably inserted between the leads of the 2 output circuits of the flowmeter amplifiers (Fig. 1). When both flowmeters were perfused with equal volumes

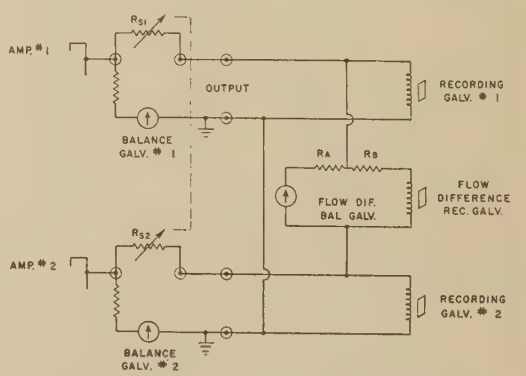

FIG. 1. Circuit diagram of flow-difference-meter used in conjunction with two 5734 bristle flowmeters. The output stages of amplifiers No. 1 and No. 2 are identical. The recording galvanometers No. 1 and 2 are also identical. The series resistances R_{S1} and R_{S2} are sections of a dual potentiometer wired in opposition. They can be made equal by insertion in a bridge circuit. Resistances R_A and R_B are determined by the current requirements of the flow-difference balance and recording galvanometers.

* Project supported by Life Insurance Medical Research Fund and the Cleveland Area Heart Society.

† Manufactured by Sigmamotor, Middleport, N. Y.

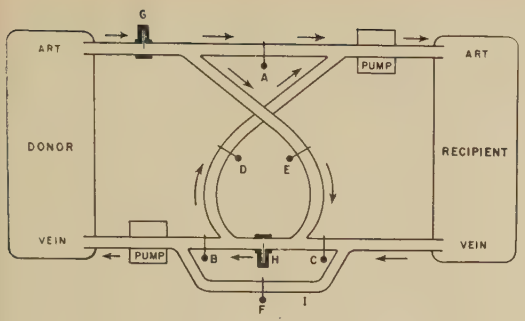

FIG. 2. Diagram of cross-circulation arranged for determining zero flow reading of flow-difference-meter, G and H: Bristle flowmeters; A, B and C: Clamps to be closed for temporary rerouting of donor to patient blood flow through both flowmeters; D, E and F: Clamps to be closed for measuring blood flow in donor to recipient and recipient to donor passages. Description in text. The 2 pumps indicated in Fig. 2 are actually one sigmamotor pump.

of blood, the D.C. output of both amplifiers was adjusted to an equal level and thus read "zero flow difference." If the flows in the 2 sides of the cross-circulation circuit were unequal, this difference was indicated by a deviation of the flow-difference-meter from its zero reading. However, due to the inherent drift characteristics of all electronic recording devices, "base line drift" in one or both amplifiers could cause a deviation of the flow-difference-galvanometer from its zero position even though flow remained equal. In other words, a flow-difference-meter cannot distinguish between electronic drift and a difference in flow. For this reason, provisions had to be made to correct for base line drift in the flow-difference-meter by checking frequently its zero reading during the cross-circulation. This was accomplished by shunting for brief periods the blood of one side of the circuit through both flowmeters, thus perfusing them in series with identical amounts of blood (Fig. 2). By closing clamps A, B and C and simultaneously opening clamps D, E and F, blood was re-routed so that flow from donor to recipient passed not only through flowmeter G, but also through flowmeter H. During this test period, flow from recipient to donor was not metered since it by-passed flowmeter H through shunt I. Since equal flows passed through both flowmeters during the shunting period, the flow-difference-meter could be adjusted to read zero if drift had occurred. Both bristle flowmeters and the flow-difference-meter were connected to mirror-galvanometers and the flow tracings were recorded on a photokymograph. 2. *Cumulative* flow difference recorder (Fig. 3). Differences of flow from donor to recipient and recipient to donor were registered by recording the changes in weight of the donor, the donor resting throughout the proceedings on scales‡ which were counterbalanced to read zero (±3 g). It was assumed, other conditions remaining constant, that an *increase* or *decrease* of the donor's weight was due to an imbalance of the cross-circulation. Two bubble traps of 125 ml each were interposed on the output sides of the pump. Throttles A and V balanced flows on the 2 sides of the circuit. The donor's weight was kept constant by intravenous infusion of blood and by a temporary change of the throttle A on the arterial side of the circuit. Blood lost from the recipient was collected and returned to the entire circuit *via* intravenous infusion of the donor. In addition, the following modifications were tested: (a) a constant suction reservoir between recipient and pump inflow on the venous side of the circuit (Fig. 3), (b) an additional suction reservoir between donor and pump inflow on the arterial side of the circuit, (c) use of 2 suction reservoirs as in b, but without scales, and (d) use of scales for both donor and recipient with and without

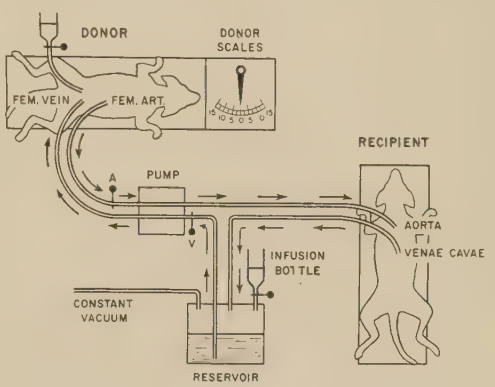

FIG. 3. Diagram of cross-circulation illustrating the position of scales and reservoir used for regulating the 2-way flow of blood.

‡ The scale used was "Speedweigh" No. 3031D zero reading scale, furnished by the kindness of Toledo Scales Co., Toledo, O.

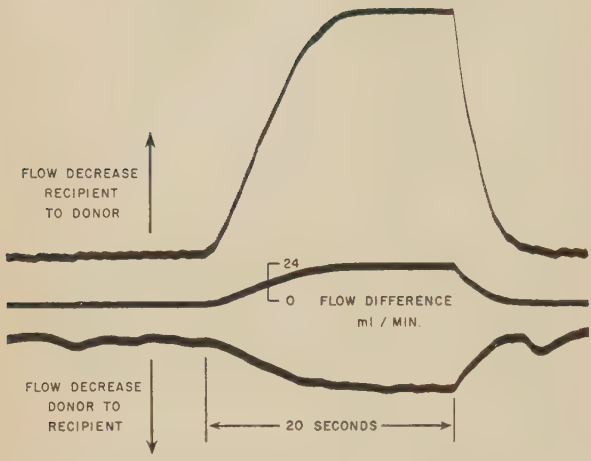

FIG. 4. Original optical record illustrating differences in mean flow rates between the 2 sides of the cross-circulation system. Description in text. The small fluctuations in the upper tracing are caused by the donor's spontaneous respiration affecting his venous return from the recipient. The larger, slower waves in the lower tracing are apparently due to fluctuations of the resistance in the arterial system of the recipient. (Reduction: ¼ of original.)

suction reservoirs. Nine *acute experiments* were undertaken under simulated operating room conditions in dogs anesthetized with 1.5 mg/kg morphine sulfate and 15 mg/kg pentobarbital. The 9 donor dogs ranged from 15 to 22 kg and the 9 recipient dogs from 13 to 17 kg in weight. Cannulation procedures followed essentially those described by Warden *et al.*(1). Donor and recipient weight changes, arterial blood pressures, and reservoir variations were continuously recorded. Through a right ventricular cardiotomy under direct vision large interventricular septal defects were created and then repaired.

Results., 1. Flow *rate* recorder: Fig. 4 depicts a record demonstrating flow changes registered with 2 bristle flowmeters in the cross-circulation circuit. The upper tracing was recorded by flowmeter (H) and the lower tracing by flowmeter (G) (see also Fig. 2). No reservoirs were used in these circuits. The middle tracing is that of the flow-difference-meter. In the left part of the record it can be seen that flow (550 cc/min) was equal in each side of the circuit, the flow-difference-meter reading zero. Flow was throttled for 20 seconds in the recipient to donor tube resulting in a reduction of the flow rate (upstroke in the upper tracing). Flow in the donor to recipient side fell subsequently. The flow-difference-meter tracing indicates by its upward movement that less blood passed from recipient donor than vice versa. The flow rate difference (galvanometer deflection 18 mm) amounted to 24 ml/min or 5% of the total flow. The sensitivity is indicated by the reflection of the donor's respiratory variations in venous return and by the fact that 1/18 of the deviation of the flow-difference galvanometer can be read on the original record, permitting an estimation of flow differences of less than 1%.

2. *Cumulative* flow recorder: Fig. 5 shows in a representative experiment the weight changes and mean blood pressures in donor and recipient during 170 minutes of cross-circulation *without the use of a reservoir*. It is noted that the donor's weight could be kept constant during the entire operative procedure by the judicious use of intravenous infusion of the donor and temporary change of the throttle on the arterial side of the circuit. Toward the end of the experiment various amounts of blood were infused intravenously into the donor without changing the throttle between donor to recipient. This was reflected quantitatively in the donor's weight gain.

A transient blood pressure fall in donor and recipient as shown in Fig. 5 occurred after the beginning of cross-circulation in 4 experiments. The fall in blood pressure may have been exacerbated by sodium pentobarbital. Similar transient decreases in blood pressure

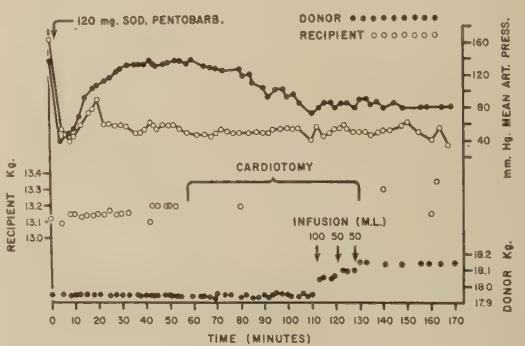

FIG. 5. Weight and blood pressure of donor and recipient illustrating the effectiveness of scales in regulating the 2-way blood flow during cross-circulation (0 to 170 min.).

at the onset of cross-circulation were recently reported by Egdahl(7) in about half of his experiments on uncontrolled cross-circulation of dogs.

The advantages of using a reservoir on the venous side of the circuit (Fig. 3) can be enumerated as follows: (1) It permitted visualization of variations in caval outflow. (2) The *flow rate* could be *directly* determined by clamping the venous outflow tube from the recipient for a few seconds and measuring the fall of the reservoir level. (3) A decrease of caval flow in the event of catheter blockage or recipient blood loss, was indicated by a fall of the reservoir level. (4) Despite venous catheter blockage or blood loss of the recipient, the donor's weight remained unchanged because of the constant pump intake from the reservoir, and (5) the degree of suction in the reservoir could be adjusted to the blood volume available in the venae cavae, thus minimizing the interferences with cavae outflow due to venous collapse(8). Use of an additional reservoir on the arterial side of the circuit gave corresponding information about the donor's output. With the use of 2 reservoirs, one on the recipient's and one on the donor's side, it was possible to balance flow accurately in the 2 sides of the circuit even without scales. This was done by equalizing the flow rates to both reservoirs and maintaining the reservoir levels constant.

Discussion. In the search for an accurate regulation of the blood flow in the two sides of a cross-circulation system, we have explored the practical value of several methods.

We believe that the use of *scales,* actually the oldest device for cumulative blood flow measurement(9-11) solves simply and accurately the problem of regulating the 2-way blood flow during cross-circulation in the operating room.

Attention is called to the advantages of a *cumulative* flow recorder over a flow *rate* recorder (*e.g.,* bristle flowmeter), particularly where the cardinal question is whether or not the donor is receiving as much blood as he is delivering. Since any blood loss of the donor has a cumulative effect, this will be clearly indicated by a weight loss. The amount of blood loss and the rate at which it is lost per unit time can be directly read. Thus, the donor on the scales acts as a cumulative flow-difference recorder.

Use of the donor scales alone, without the interposition of reservoirs, gave enough information to maintain the donor normovolemic. From a practical standpoint, the advantages of the use of one reservoir or even of the use of 2 reservoirs, which permit the exact balancing of flow rates without scales, are vitiated by the simplicity of donor weight recording. Additional recording of the weight of the recipient gave no significant assistance in estimating his blood loss which is visible and can be measured directly.

It is apparent that with the use of 2 bristle flowmeters instantaneous and minute changes in the flow rates can be measured. However, it should be pointed out that the bristle flowmeter (just as any other electrical flow *rate* recorder, such as the electromagnetic flowmeter, Potter turbinometer, Rotameter, bubble flowmeter, etc.) cannot register directly a cumulative blood loss from the donor. Though this could be calculated from a record taken over a certain time interval, such a procedure would be impractical in an operating room. Moreover, it must be noted that the handling of all electronic flow *rate* recorders offer a host of technical difficulties which should not be underestimated when these instruments are used outside the experimental laboratory.

Summary. In acute experiments on dogs 2 types of flowmeters were compared for regulating the 2-way flow of blood in donor cross-circulation during intracardiac surgery. (1) By arranging 2 flow *rate* recorders (5734 bristle flowmeters) to act as a *flow-difference-meter,* differences in the volume flow of blood from donor to recipient and vice versa could be recorded. This method appears, however, rather complicated for use in the operating room. (2) A *cumulative* flow recording method was found to be simple and reliable. For this purpose, the donor rested on zero reading scales which acted as a cumulative flowmeter indicating blood inflow and outflow from the donor. With the aid of the scales, blood loss from the donor could be accurately measured and replaced by intravenous infusion. For

practical purposes it is felt that continuous weighing of the donor provides the best method for insuring equal bidirectional flow in cross-circulation under operating room conditions.

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Hemagglutination Test for Detection of *Candida albicans* Antibodies in Rabbit Antiserum. (21738)

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A serologic response of humans and animals to *Candida albicans* is easily demonstrated by an agglutination test using washed, heat-killed organisms. Test tube methods(1) and slide agglutination technics(2) have been described in which cross reactions occur between *Candida albicans, Saccharomyces* and *Hansenula*(3). The present report outlines procedures for a more specific and easily read hemagglutination test to detect antibodies to *Candida albicans* in rabbit antiserum.

Materials and methods. Several strains of *Candida albicans,* freshly isolated from patients, were used. Stock strains, because of their propensity to change biochemically were avoided(4). A strain of *Saccharomyces cerevisiae* isolated from Fleischmann's yeast was also used. The *hemagglutinating antigen* was prepared from organisms grown on Sabouraud's glucose agar. Each strain was planted on 8 Roux bottles, each containing 200 ml of medium, and were incubated at 34°C for 36 hours. The growth was removed by washing the agar surface of each bottle with approximately 5 ml of sterile saline. Sterile glass beads were added to the saline suspension which was shaken for 30 minutes on a paint homogenizer. The extract was centrifuged at 3,000 rpm for 20 minutes and the sediment was discarded. The opalescent supernate was removed and adjusted to 10% sodium acetate and 1% acetic acid, and finally precipitated with 1½ volumes of 95% alcohol. The precipitated material was allowed to stand at 4°C for 12 hours and then centrifuged and reconstituted to original volume in veronal buffer.* The pH of the extract was adjusted to 7.3 with 0.1 normal sodium hydroxide. Both *sheep red cells* and *human type "O"* cells were tested. Aliquots of both types were washed 3 times with veronal buffer and 0.1 ml of the packed cells was added to 4 ml of the undiluted extract from the organisms. The mixture was incubated at 37°C for 2 hours and shaken every 15 minutes. After sensitization, the cells were spun down at 1000 to 1500 rpm, washed 3 times in fresh veronal buffer and finally made up to a 0.5% suspension. *Albino rabbits* weighing approximately 2 kilo were immunized with *C. albicans* and *S. cerevisiae.* A saline suspension corresponding to a number 6 McFarland nephelometer tube was injected intravenously. Three consecutive, daily intravenous injections of 0.1, 0.2

* Veronal buffer: stock solution, 85 g NaCl; 5.75 g 5, 5 diethyl barbituric acid and 3.75 g Na-5 5 diethyl barbiturate. H_2O to final volume 2000 ml. Working solution, Stock diluted 1:5 with water, pH 7.3-7.4.

TABLE I. Comparison of Serologic Activity of a Candida Yeast Cell Antigen and a Candida Hemagglutinating Antigen in Rabbit Antiserum to Both *C. albicans* and *S. cerevisiae*.

Antigen	Antiserum (titer)* *C. albicans*	*S. cerevisiae*
C. albicans yeast cells	1:5096	1:128
C. albicans hemagglutinating antigen (sheep or type "O" cells)	1:5096	—

* No activity was noted in normal rabbit serum.

and 0.5 ml were made the first week; 0.5, 1.0 and 1.0 ml the second week; and 1.0, 1.0 and 1.0 ml the third week. The animals were exsanguinated by cardiac puncture 7 days after the last course of injections. The rabbit *sera* to be tested were first inactivated for 30 minutes in a 56°C water bath. In order to absorb possible non-specific antibodies to normal red cells, 0.1 ml of packed red cells was added to 1 ml of serum and the mixture incubated for 10 minutes at 37°C and refrigerated for 4 hours at 4°C. The cells were removed by centrifugation. Four-tenths ml of serial dilutions starting with 1:4 made in veronal buffer were added to standard Wassermann tubes. An equal amount of sensitized 0.5% red cells was added and the tubes thoroughly shaken. Controls consisted of normal red cells and immune serum, and antigen plus veronal buffer. The test was incubated for 1½ hours at 37°C. The clumping of red cells at the bottom of the tubes indicating a positive reaction was read according to the standards given by Stavitsky(5). The tubes were shaken a second time and the final result recorded after incubation at room temperature for 24 hours. A test tube, *yeast cell agglutination* was set up with similar quantities, using thoroughly washed heat-killed organisms. The antigen was adjusted to a density reading of .410 at a wavelength of 420 mμ on a Coleman junior spectrophotometer.

Results. As shown in Table I, rabbit *Candida albicans* antiserum agglutinated both sensitized sheep and human type "O" cells. The titers in the Candida hemagglutination test were equivalent to those obtained in the direct yeast cell agglutination; however, the Candida sensitized red cells failed to cross react with Saccharomyces antiserum, which nevertheless agglutinated the Candida yeast cells at a titer of 1:128. The same pattern of activity was noted in each of three different rabbits immunized with Candida or Saccharomyces. The sensitizing capacity of these extracts was optimal only if used undiluted (Table II).

Extracts prepared from either *Candida albicans* or *Saccharomyces cerevisiae* were inactive in the test if not first precipitated by 95% alcohol. Likewise, inactivity was noted if the alcohol precipitated material was passed through a Seitz filter. Removal of protein in the extract by Sevag's chloroform-butyl alcohol method(6) did not alter the sensitizing ability of the extract, suggesting that the material was not protein but possibly polysaccharide. The active alcohol precipitated material was stable for at least 1 month at 4 to 10°C. Sheep cells appeared preferable to human type "O" cells since the positive agglutination pattern, observed after 1½ hours incubation time with "O" cells, was dispersed when the tubes were shaken and allowed to stand for 24 hours. The stronger sheep cell agglutination was retained after the 24 hour period.

Discussion. Norris and Rawson(7) reported a considerable number of cross reactions in human sera between *Saccharomyces* and *Candida albicans* yeast cell antigens in a slide agglutination test. They noted a tendency for sera either to have no agglutinins for Candida or Saccharomyces or to have them for both organisms. In this laboratory, using the test tube method and human sera positive for Candida agglutinins, cross reactions with Saccharomyces have been noted but are relatively few. In Candida or Saccharomyces rabbit antiserum cross reactions between these two organisms are easily dem-

TABLE II. Antigen Titration of Candida Hemagglutinating Antigen in Candida Rabbit Antiserum.

Dil. of Candida extract to sensitize cells	Candida antiserum (titer)
Undiluted	1:5096
1:2	1:2048
1:4	1:128
1:8	—

onstrated. Moreover, the extent of cross reaction of Candida in Saccharomyces antiserum depends upon the amount of cross reacting surface antigen present in the particular strain used for antigen(8). In addition to the known cross reactions occurring in Candida antiserum with other yeast-like organisms, the previous tests have been criticized as difficult to read because of spontaneous agglutination(2). The Candida hemagglutinating extracts in the present report were shown to contain varying amounts of cross reacting Saccharomyces antigen by precipitin tests. Nevertheless, red cell antigens prepared from extracts of several freshly isolated strains of Candida failed to react in Saccharomyces antiserum which agglutinated the Candida yeast cells to a 1:128 titer. It appears that a hemagglutinating antigen prepared from Candida will not react with cross reacting antibody produced by Candida or Saccharomyces, therefore a positive reaction obtained using this antigen should indicate antibody to the Candida group.

In studies on a hemagglutinating antigen derived from *P. tularensis,* Wright and Feinberg(9) reported that alcohol precipitation of a sonic vibrated, saline extracted and lyophilized material was necessary to eliminate impurities which caused spontaneous agglutination of the antigen. In the present work, alcohol precipitation was also essential in preparing the Candida antigen, making the difference between obtaining a positive or negative test. Further studies are in progress using human sera.

Summary. Saline washings from *Candida albicans* yeast cells have been found to yield an active hemagglutinating substance which does not appear to be protein in nature. A Candida yeast cell antigen cross reacted with Saccharomyces rabbit antiserum. On the other hand, a Candida hemagglutinating antigen, equally effective in the homologous test, eliminated the cross reaction with Saccharomyces rabbit antiserum.

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Nor-epinephrine Cells of Adrenal Medulla Following Hypothermia and Unilateral Adrenalectomy. (21739)

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Many investigations have revealed morphologic and chemical alterations in the adrenal cortices of animals subjected to stress. It has also been observed that the adrenal medulla plays a role in the response to such stimuli, being morphologically characterized by a discharge of chromaffin granules. This alteration has been interpreted as indicating epinephrine release. Since many of these studies were performed prior to the identification of nor-epinephrine within the adrenal medulla it is not surprising to find little mention concerning the role of this substance in the response to stress. In addition, the conventional methods utilized for the demonstration of chromaffin fails to differentiate between these 2 medullary substances. Recently a reliable and easy method has been described allowing

TABLE I. Evaluation of Number of Nor-epinephrine Cells Counted.

	Group I		Group II	
	A (prior to cold)	B (after cold)	AC (anesth. cont.)	BC (after 1 hr)
No. rats	15	15	9	9
Means, cells/1 unit area	2.78 ± 1.47*	3.30 ± 1.22		2.56 ± 1.05

* Based on means of A and AC.
p value between A and B, .3; between A and BC, .7; between B and BC, .2.
1 unit area = .1 mm.

for the identification of nor-epinephrine within adrenal medullary cells(1). It was therefore considered advantageous to explore possible morphologic alterations of these cells in rats subjected to extreme cold as well as unilateral adrenalectomy.

Materials and methods. 24 adult white Sprague-Dawley rats weighing between 225 and 275 g were divided into 2 groups. Group I consisted of 15 animals which were subjected to unilateral adrenalectomy(A) under ether anesthesia. Immediately following this procedure each animal was immersed in an ice bath at 1°C. Rectal temperatures were maintained at 18°C-23°C. The animal was then sacrificed and the remaining adrenal removed(B). Group II consisted of 9 animals and were utilized as anesthesia controls. Unilateral adrenalectomy (AC) was performed under ether anesthesia as in Group I. The remaining adrenal (BC) was removed one hour later. All animals were maintained on a stock diet of chow checkers and water *ad libitum* and were not fasted prior to experimentation. All *adrenals* when removed were immediately bisected. One-half was placed in 10% potassium iodate for 48 hours as described by Hillarp and Hökfelt(1) for the demonstration of nor-epinephrine. These were then transferred to 10% neutral formalin for 24 hours. Frozen sections were cut at 10 microns and counterstained lightly with hematoxylin. The remaining portion of adrenal was fixed for 4 days in Orth's fluid, dehydrated and infiltrated with paraffin in the usual manner. Sections were stained with hematoxylin and eosin.

Results. The cells of the medulla containing nor-epinephrine were distinguished by their closely packed cytoplasmic granules which appeared brown with the periodate en bloc technic. These cells were arranged in small clusters within the medulla being separated by groups of cells with non-reactive cytoplasms. In order to eliminate any subjective impression concerning alterations in these cells actual counts were performed including all cells within the available medullary tissue present in the section. Each actual cell count obtained was adjusted on the basis of a unit area of 1 which was derived from the product of the area counted vertically and horizontally with a micrometer eyepiece divided by 150 which represented a convenient, arbitrary area for these determinations. The mean and standard deviation of the mean of the counts in each group was then determined. P values for statistical significance between A (adrenal prior to cooling) and AC (adrenal from anesthesia control) and B (adrenal following cooling); and B and BC (adrenal one hour after anesthesia) were then obtained by utilizing Fisher's t(2). From Table I it becomes apparent that no statistically significant difference can be discerned in the number of nor-epinephrine cells of the adrenals obtained prior to cooling and following immersion into the ice water bath. Similarly no deviation of statistical significance could be found in nor-epinephrine content in the adrenals of the animals subjected only to unilateral adrenalectomy utilizing ether anesthesia and followed by removal of the remaining adrenal without exposure to cold. Variation in the intensity of the reaction for nor-epinephrine was not evident.

The chromaffin reaction observed after Orth's fixation revealed practically all of the medullary cells to possess brown cytoplasms. Although actual counts were not performed on the total chromaffin reaction it might be stated that alterations if present were not of

the magnitude which allowed for recognition by inspection. There did not appear to be any variation in the intensity of the chromaffin reaction in any of the groups studied.

Discussion. It has been noted previously from pharmacologic studies that nor-epinephrine unlike epinephrine has little pituitary stimulating function or eosinopenic activity (3,4). On the other hand, its pressor activity has been estimated as being of greater magnitude. That its release might be a factor in the early hypertension noted in the alarm reaction could not be substantiated by the results of this study. Although the release of epinephrine has been considered an important phase of the reaction to stress by some(5), morphologic evidence of this phenomenon was lacking under the condition employed in this study. Selye(6) considers the chromaffin response in stress to be of significance perhaps only in the first few minutes of the alarm reaction and that it is questionable if it is of importance at any other time during the course of the general adaptation syndrome. In the light of our observations this would imply that rapid regranulation of the adrenal medulla occurs although suggestive evidence to the contrary exists(7). Gordon(8) has noted decreased cortical ascorbic acid following histamine injection, exposure to cold and hypoglycemia in rats subjected to apparently complete adrenal demedullation. His observations certainly minimize the role of the adrenal medulla in stress.

The exposure to cold employed in this experiment was of hypothermic degree. In the light of the recent observations by Egdahl, Nelson and Hume(9) who observed diminished corticoid secretion in hypothermic dogs an explanation for the lack of chromaffin response in the animals exposed to cold would be consistent with their contention of lack of alarm in hypothermia. However, the stress associated with unilateral adrenalectomy and ether anesthesia was also found not to be associated with alterations in the chromaffin or nor-epinephrine reactions.

Summary. Rats subjected to hypothermic levels of cold failed to reveal any alteration in the nor-epinephrine or total chromaffin reaction of the adrenal medulla. Similarly no alterations in these reactions were observed following surgical trauma incident to unilateral adrenalectomy under ether anesthesia.

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Effect of Hypophysectomy on Survival after X-Irradiation.* (21740)

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The role of the hypophysis in conditioning survival after total body X-irradiation has not been defined clearly. According to Finerty *et al.*(1) the failure of hypophysectomy to modify the protection afforded by parabiosis "tends to eliminate the role of the hypophysis and its dependent endocrine organs as critical factors in recovery from irradiation sickness." Other studies, however, indicate that sensitivity to irradiation may be increased by pituitary ablation. With 20 hypophysecto-

* Supported in part by grants from the University of Michigan-Memorial Phoenix Project, the National Institutes of Health and the Upjohn Co.

mized rats irradiated at one dose (750 r), Patt *et al.*(2) observed evidence of increased X-ray toxicity. Kohn(3) stated that the LD_{50} of adult hypophysectomized rats occurs at a dose of about 650 r as contrasted to 750 r for non-hypophysectomized rats. He submitted no data in support of this conclusion. The purpose of this investigation is to resolve these differences by studying the survival and time of death in a large number of hypophysectomized and non-hypophysectomized rats irradiated over a considerable range of dosages.

Procedure. Female Sprague-Dawley rats weighing 150-190 g were used. Some were hypophysectomized, the completeness of removal being ascertained by failure to gain in body weight during a 3 week post-operative period. Those which increased in weight were discarded. Early in the fourth post-operative week, hypophysectomized and non-hypophysectomized rats received whole-body X-irradiation in groups of 8. It was administered by a Keleket Theron X-ray therapy machine operated at 200 KV, and with ½ mm Cu plus 1 mm Al added filtration. The distance from the center line of the X-ray tube to the table on which the animal container rested was 110 cm. The animal container was constructed of sheet Lucite ¼ in. thick and had the form of a flat cylinder divided radially into 9 equal compartments. The X-ray intensity was calibrated by giving a Victoreen condenser r-meter chamber a timed exposure while located inside the animal container in a position which received an average intensity of irradiation. The dose rate was indicated by a Victoreen rate meter and held constant at about 11 r/minute by varying the current of the X-ray tube over a range of 20-25 ma. The doses employed on the 542 non-hypophysectomized and 433 hypophysectomized rats are shown in Table I.

TABLE I. Number of Animals Used at Various Doses of X-radiation.

Non-hypophysectomized		Hypophysectomized	
Dose (r)	No.	Dose (r)	No.
575	31	0	56
600	31	375	30
625	31	400	31
650	39	425	39
675	94	450	31
700	96	475	59
725	63	500	31
750	62	525	32
775	32	550	56
800	32	575	32
825	31	600	28
		625	8

The rats were transported from their regular quarters to the site for irradiation in a specially insulated box, the internal temperature of which did not vary more than 0.1°C/min. when empty. A group of 56 hypophysectomized rats did not receive any irradiation but were observed in order to determine the number which might be expected to die during the 30-day period of observation because of other causes. After irradiation, the animals were kept in groups of 4 or 8 according to the

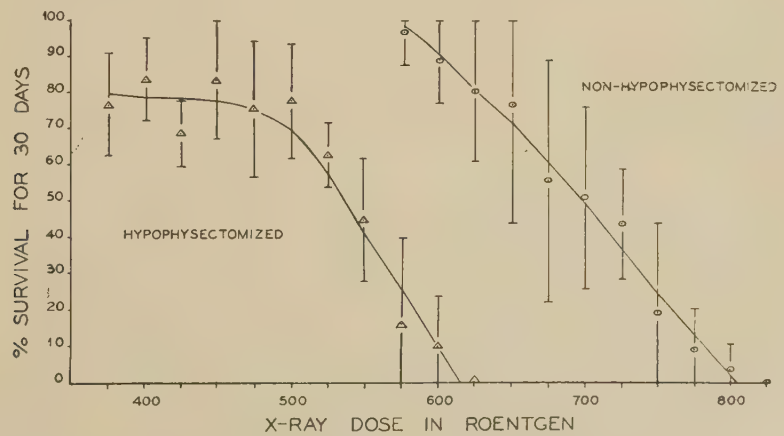

FIG. 1. Survival curves for hypophysectomized and non-hypophysectomized rats. Vertical bars are standard deviations based on variation in survival among individual groups of 8 rats which were irradiated at each dose level.

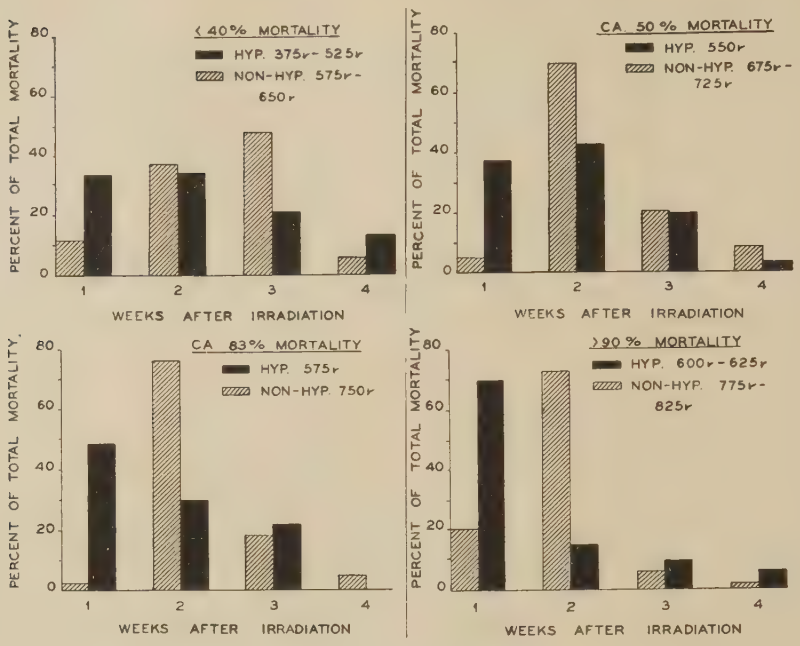

FIG. 2. Graphs comparing time of death in hypophysectomized and non-hypophysectomized groups with similar percentages of mortality. Hyp. = hypophysectomy.

size of the cage. They were inspected one or more times daily during a 30-day period of observation in order to identify and record the animals which died. The diet consisted of Purina Laboratory Chow supplemented twice weekly with greens, oranges and a vitamin concentrate. The temperature of the animal quarters was maintained at 75 ± 3°F.

Observations. In the control, non-irradiated hypophysectomized group, 3.6% of the rats died during the 30-day period of observation which began 21 days after hypophysectomy. If the survival curve for hypophysectomized-irradiated rats (Fig. 1) is corrected to this extent, between the 0 and 70% levels of survival an average of about 157 ± 13 r separates this curve from that for the non-hypophysectomized-irradiated animals. Thus, the LD_{50} was about 550 r for the hypophysectomized and 700 r for the non-hypophysectomized rats. To this extent, removal of the hypophysis increased the sensitivity of rats to irradiation. At doses below 500 r the survival curve for the hypophysectomized group flattened and at the lowest dosage studied, 375 r, did not allow 100% survival even after correction for the rats which would have been expected to die from other causes.

Fig. 2 demonstrates that hypophysectomized rats tended to die earlier after irradiation than non-hypophysectomized rats. This was significant when the groups were selected for comparison on the basis of similarity in percentage of mortality rather than on the basis of radiation dosage. Furthermore, the earlier death in the hypophysectomized group was more marked at the higher doses of radiation.

Discussion. These results demonstrate that the hypophysectomized rat has a reduced capacity to withstand total body X-irradiation and supports the conclusions of Patt *et al.*(2) and Kohn(3) which were obtained in less extensive investigations. Kohn reported the LD_{50} to be about 100 r lower in hypophysectomized than in non-hypophysectomized adult rats. This is somewhat less than the difference observed in our study, which may be related to the different strains or sizes of rat used, or more likely to the period elapsing between the time of hypophysectomy and irradiation. Kohn did not give the post-operative interval which he used. It is probable that a longer post-operative interval in our study would have increased the sensitivity of

hypophysectomized rats still more since the anemia which follows pituitary ablation does not reach its maximal severity within 3 weeks.

The change in the shape of the survival curve for hypophysectomized rats at low doses of X-irradiation has significance in demonstrating that this portion of the curve cannot be extrapolated from a few points obtained at higher doses. An explanation for the shape of this portion of the curve is not evident but must be related to factors which are unknown or not controlled. Further investigation of survival at still lower doses did not appear promising at this time.

In normal mice, the time of survival after irradiation with the doses used in our study tends to be inversely related to the dose of radiation(4). In our study, few non-hypophysectomized rats died before the second week until an LD_{100} was approached. The tendency toward earlier death in hypophysectomized rats was suggested by the observations of Patt *et al.*(2) and clearly evident in the data of Kohn(3) for immature rats. This period corresponds with the early gastrointestinal phase of the radiation syndrome. Undoubtedly, this response results from the severe structural and functional deficiencies which arise in the gastro-intestinal tract and its associated glands after pituitary ablation (5). The relationship of hypophysectomy to death during the hematological phase of the syndrome is discussed elsewhere(6).

Clarification of the manner in which the pituitary gland influences resistance to irradiation will require additional study of the influence of replacement therapy on hypophysectomized rats. Administration of somatotropin to non-hypophysectomized irradiated rats with(7) or without(8) concurrent treatment with antibiotics appears to have no influence on survival. Consideration must be given to the possible mediation of this function by the other endocrine glands which are dependent on the anterior hypophysis. Many reports are available on the effect of overdosage with the hormones elaborated by these glands on survival of animals which do not already suffer from a primary endocrine deficiency. These studies offer little help toward solution of this problem because usually the therapy itself is damaging. On the other hand, thyroidectomy increases the mortality of rats after a single high dose of total body X-radiation(9). Although adrenalectomy is reported to be without effect(10), others have established that adrenocortical insufficiency increases sensitivity(11,12) in this species. It is probable that thyroid and adrenocortical deficiency, secondary to pituitary removal, is an important factor in explaining the increased sensitivity of hypophysectomized rats to total body irradiation.

Summary. Hypophysectomy of 433 adult female rats reduced their capacity to survive total body X-irradiation as compared with the response of 542 non-hypophysectomized rats. The 0 to 70% levels of survival were obtained at doses which averaged 157 r lower for hypophysectomized than for non-hypophysectomized rats. At doses ranging from 375 to 500 r the percentage of survival of hypophysectomized rats did not change but remained at about the 80% level. In the absence of the pituitary gland, rats tended to die earlier after irradiation.

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Alteration in Fibroblasts Treated with Steroids in a Perfusion Chamber.* (21741)

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The study of the response of connective tissue cells *in vitro* to steroid compounds has been hampered by the fact that these substances are generally too slow to act at concentrations which, in conventional tissue cultures, are low enough not to be too irritating to the cells. Since conventional tissue culture methods are limited to a relatively short period of undisturbed optimal growth, it has been difficult to determine which are the effects truly accountable to the physiological activity of the steroid and which to the alteration of the medium. This seems to be the reason for the lack of correlation between the data obtained from studies carried out *in vivo* and *in vitro*.

Buchsbaum(1) has developed a perfusion technic by means of which it is possible to maintain groups of cells for long periods and at the same time to keep the cells under continuous observation at high magnification. The apparatus provides a means by which the response of cells to lower concentrations of some adrenal hormones may be studied over extended periods.

Materials and methods. The steroids used in this investigation were: cholic acid, 11-desoxycorticosterone glycoside (DOCG),† 11-desoxycorticosterone acetate (DOCA), pregnenolone, estradiol, and 17 hydroxy-11-desoxycorticosterone acetate (Compound S). All have limited solubility but are soluble to the extent required by these technics. The cells used were chick and mouse *embryo fibroblasts*. The chick fibroblasts were obtained from the periosteum of the long bones of 9-day-old chick embryos. The mouse fibroblasts were obtained from explants from the long bones of embryos of about the 15th to 17th day of gestation. The stock cultures were maintained by subculturing the strains in sitting drops of a mixture of plasma and embryo extract(2). After the initial explantation, the cultures were transferred several times to increase the uniformity of cell type. When sufficiently homogeneous growth was obtained, the tissue cells were explanted into a mixture of heparinized plasma and a drop of embryo extract on a round No. 0 coverslip. The coverslip was then put on a drop of sterile water on a heavy coverslip and sealed over a Maximow slide. The tissue fragment was allowed to remain in the double coverslip preparation for about 24 hours. A sufficient outgrowth occurs in this time. At the end of the 24-hour period the round coverslip bearing the culture was removed and placed in a perfusion chamber(1). The perfusion chamber was then placed on a phase contrast microscope stage in a hotbox maintained at 37.5°C. The chamber was attached by two capillary polyethylene tubes, one leading to a reservoir containing culture medium, the other serving as a drain. Fluid medium was delivered to the perfusion chamber by means of a pump (Fig. 1)‡ at the rate of 1 ml per hour. This pump differs from Woerner's(3) in that his is intermittent, while ours is continuous thereby better simulating capillary flow. The syringe was filled from a reservoir (which served to equilibrate the medium with oxygen and carbon dioxide). The cells were commonly perfused in the chamber one hour in 3 throughout a 24-hour period.

The *control medium* consisted of 80% Tyrode's solution and 20% horse serum. The chamber was perfused with the control medium for 24 to 36 hours which established

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† DOCG was obtained through the courtesy of Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, N. J.

‡ Developed by E. Perkins, Cyclotron Machine Shop, University of Pittsburgh.

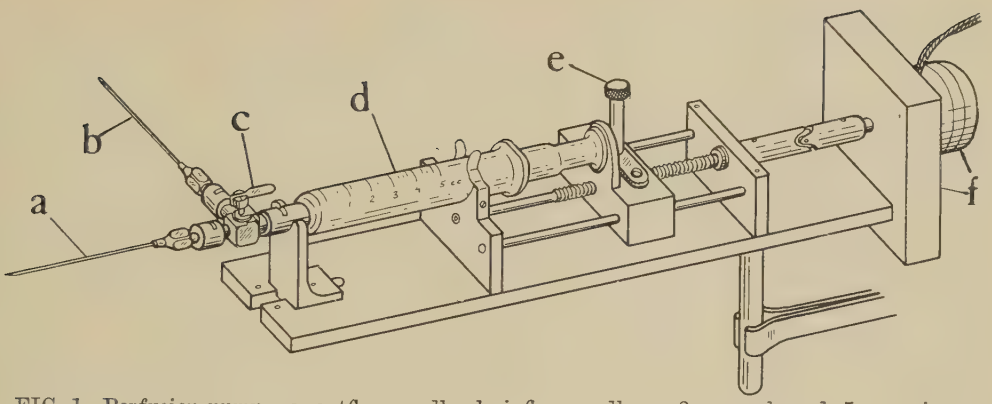

FIG. 1. Perfusion pump. a, outflow needle; b, inflow needle; c, 2-way valve; d, 5 cc syringe; e, pressure plate adjusting screw; f, synchronous motor and gear box.

suitable conditions for the cells. The *steroids* were added to the perfusion fluid. A weighed quantity of the steroid was dissolved in 80 ml of Tyrode's solution. The resulting solution was sterilized by filtration through a Seias filtering candle. Twenty ml of horse serum were then added to the Tyrode-steroid solution. Before the steroid-containing medium was perfused over the cells, they were photographed under oil-immersion phase microscopy to record the appearance of the cells at the beginning of the experiment. The same cells were photographed again at various intervals to show changes produced. At the end of the experiment, photomicrographs were made of a large number of cells to show whether or not the changes observed were frequent occurrences or isolated phenomena. In some cases cinephotomicrography was used to record the behavior of untreated and treated cells. Nuclei were measured at intervals with an ocular micrometer.

Results. DOCG, DOCA, and Compound S produced marked changes in chick fibroblasts. The nucleus and heterochromatic material in the nucleus became greatly enlarged in the treated cells (Fig. 3) over that in the controls (Fig. 2). In addition, there was a large increase in the cytoplasmic volume of the cells. The mitochondria appeared to remain normal but increased greatly in number. The spherical mitochondria appeared to be more numerous and formed chainlike aggregations alternating with large and small particles. After 55 hours of perfusion with any one of these steroids, there seemed to be an exaggerated tendency for the cells to lay down fibers. The nuclei of cells treated with 35 μg of DOCA per ml increased 320% in volume; those treated with Compound S increased 250% in volume.

Measurements of the nuclei of cells treated with 70 μg per ml of Compound S show that the most rapid increase in nuclear size is after 36 hours. The increase continues even after the cells are returned to a normal medium. Maximum size is reached in about 72 hours. Treatment with DOCA produced comparable results. Compound S appeared to be less toxic than either DOCA or DOCG; equivalent concentrations of Compound S could be continuously perfused over the fibroblasts for a greater length of time before the cells began to exhibit such degenerative changes as lipid vacuolation.

With each of the 3 steroids mentioned there was a tendency for the fibroblasts to accumulate lipid material in the cytoplasm. This lipid material is voided from the cytoplasm by extrusion of the vacuole along with a bit of cytoplasm from the trailing portion of the cell as it moves along. This process seems to differ from fiber formation. The fibers, as noted by Ragan(4), seem to be spun from a large part of the surface of the cell while the lipid seems to be extruded from the temporary posterior area of the fibroblast.

The movement of the treated fibroblasts is greatly decreased. This is believed to be a mechanical factor owing to the great size of

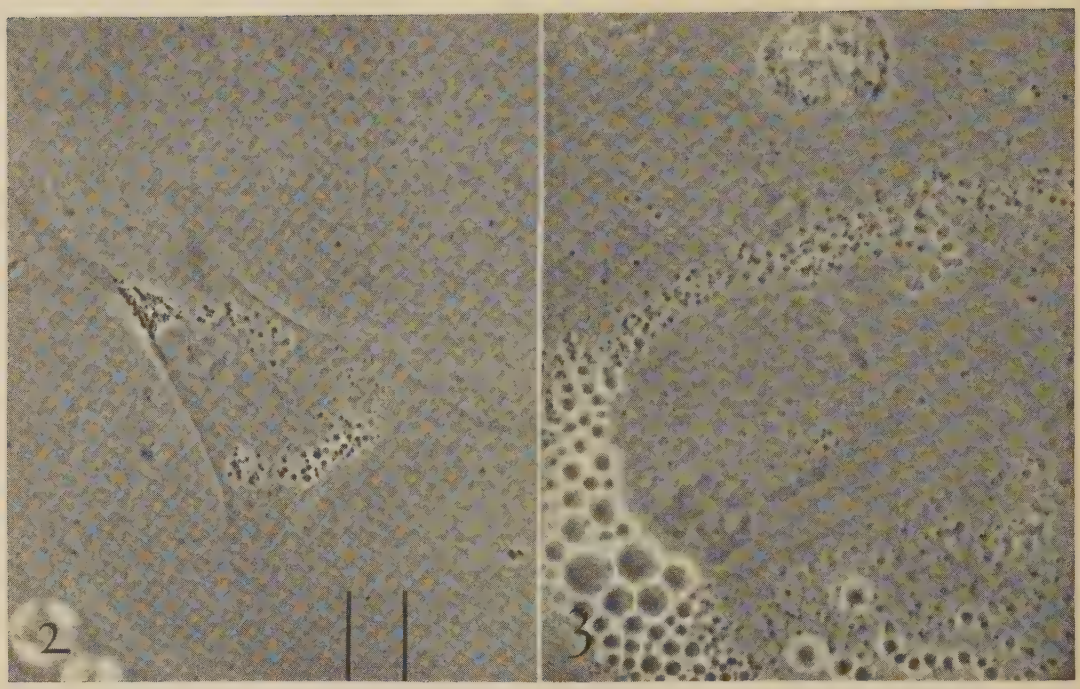

FIG. 2. A normal chick fibroblast 36 hr in perfusion chamber photographed just prior to treatment with desoxycorticosterone glycoside. (Distance between 2 black lines is 10 μ.)

FIG. 3. Portion of a cell from the same preparation showing the nucleus and adjacent cytoplasm after 72 hr perfusion with medium containing 70 μg/ml of desoxycorticosterone glycoside. Note the great increase in cellular size. (Same magnification as Fig. 2.)

the treated cells. Time-lapse cinephotomicrographs of the fibroblasts treated with Compound S reveal that the movement within the cells is nearly normal. The mitochondrial movement is quite normal despite the great increase in the total numbers. The nucleus appears somewhat less mobile. This is probably due to its great size.

Reversibility of these cellular effects presents another problem. If cells treated with DOCA, DOCG, or Compound S are returned to a normal control medium there is no change even on long perfusion. Unlike certain other effects(1) the DOCA-effect seems irreversible under these conditions.

Stained with hematoxylin, the treated cultures show alterations in the configuration of the cultures. Low power photomicrography of the fibroblasts treated with Compound S and DOCA reveal a change in the periphery of the cultures. The fibroblasts lose their regular outlines in favor of a more diffuse configuration. There is a tendency for the cells to be more isolated rather than closely packed as in the controls.

Oil-immersion photomicrography of the stained DOCA-treated cultures show a large number of unusual nuclei. In the majority of the cells treated for 2 to 3 weeks with DOCA the nucleus appears kidney-shaped and usually has a large vacuole in the indentation. *Time-lapse photomicrographs* of the response to DOCA reveal that the increase in nuclear size becomes noticeable within 4 hours after starting the treatment. This increase continues for about 72 hours. There is also an increased rate of ameboid movement for the first 12 hours. After this time the movement slows down considerably, until after 36 hours, the cells become quite sessile.

Apparently DOCA also exhibits a threshold phenomenon; concentrations of 70, 35 and 12 μg per ml all elicit the same response in the same approximate time interval.

Both chick and mouse fibroblasts give identical responses to DOCA, except for the more rapid rate of response of mouse fibroblasts. A maximal response could be expected in from

28 to 36 hours after the steroid was first perfused over the cells.

DOCA was found to have no effect upon chick epithelium in concentrations that were high enough to cause a maximal response in fibroblasts. In mixed cultures of fibroblasts and epithelium perfused with DOCA, the fibroblasts showed a general response while adjacent epithelium appeared unchanged. DOCG, DOCA, and Compound S evoke a general pattern of cellular response which appears to be a unique feature of connective tissue cells *in vitro*: increase in nuclear and nucleolar size, increased fiber formation, and increase in cytoplasmic volume.

Pregnenolone, cholic acid, and estradiol were found to have no effect upon the fibroblasts even after long perfusion with relatively high concentrations.

Discussion. This investigation also demonstrates that this method of perfusion is applicable for the study of the action of hormones on cells. The degree of response on a cellular level is much greater than that which could be achieved had the chemical substances been applied to the whole organism.

Some cellular changes observed *in vivo* seem to be closely related to those observed here. Taubenhaus(5) reports that DOCA caused an increase in size and produced a stellate appearance in fibroblasts of granulation tissue of wounds. In addition, the fibroblasts had prominent nucleoli and produced large amount of collagen. A similar response *in vitro* is shown in the data of the present investigation. Taubenhaus also noted the presence of a great number of mitotic figures indicating rapid division of the cells. Since mitosis is found only rarely in preparations perfused with DOCA, DOCG, or Compound S, other hormone factors seem to be operative at this level.

Fibroblasts from granulation tissue are much smaller than those found in our perfusion studies as deduced from a comparison of photomicrographs and descriptions. This may be explained by the fact that fibroblasts in the present investigation represent an extreme response because much larger dosages were used and possibly because certain organismal factors such as the pituitary hormones are absent *in vitro*. Fibroblasts from granulation tissue have been shown to lose their regular outline and orderly arrangement in favor of a more diffuse appearance when treated with DOCA (6). Low power photomicrographs of DOCA-treated fibroblasts show a similar tendency *in vitro*.

The suppression of healing of wounds by the estrogenic hormones introduced into the whole animal also has been reported(6). This may be due to an indirect influence on metabolism rather than a direct action of estradiol on fibroblasts because estradiol appears to have no effect on fibroblasts according to the observations described here. Taubenhaus(7) demonstrated an increase in the formation of collagen in healing wounds of animals treated with DOCA. The possible importance of DOCA in collagen diseases has been reported (8). This leads the authors to believe that the fibers present in cultures of DOCA-treated fibroblasts were due to the increase in collagen-formation by the cells.

Cornman(9) reports the inhibition of fibroblasts by concentrations of DOCA of 20 μg per ml and higher in conventional tissue cultures. In addition, the same author(10) demonstrated the selective damage to fibroblasts in mixed cultures of fibroblasts and epithelium in roller tubes. Gillette and Buchsbaum(11) found a stimulation of chick fibroblasts in drop cultures by low concentrations of DOCG. A maximal stimulation was observed at 7 μg per ml of DOCG. The concentrations of steroids used in our perfusion apparatus are higher than those cited above(10, 11), and on the basis of these experiments would be expected to show greater toxic effects; but, because the cells are maintained under better physiological condition in the perfusion apparatus used here, there is no accumulation of metabolic by-products which might alter the response of the cells to the hormones. A situation more analogous to conditions *in vivo* is produced.

Summary. The response of chick and mouse fibroblasts to adrenal and non-adrenal steroids was studied in a perfusion apparatus. Cholic acid, estradiol, and pregnenolone induced no changes in fibroblasts. DOCA, DOCG, and Compound S caused a great in-

crease in the size of the cells. The nucleus and heterochromatic material within the nucleus were greatly increased in size. There was an increase in the number of mitochondria and a tendency to lay down large numbers of fibers.

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Breeding of Sex-reversed Males of *Xenopus laevis* Daudin.* (21742)

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For several years it has been known that under the influence of estradiol(1,2) and of estradiol benzoate(3) genetic males of *Xenopus laevis* differentiate like the genetic females. They acquire normal ovaries and sometimes also normal oviducts, the latter character depending on dosage levels and duration of treatments. By contrast, androgenic hormones interfere little with the development of the ovaries of genetic females; this is the more remarkable, as the treatments cause in both males and females a precocious development of the male secondary sex characters. Immediately following metamorphosis, the tiny female toads as well as the males acquire the characteristically male copulatory pads on arms and hands, and exhibit vigorous copulatory instincts. It is most impressive to find normal ovaries upon dissection of such a small animal (15 mm as against over 45 mm of mature adults) which one just had observed expertly clasping a brother or sister.

Since Xenopus reproduces easily under laboratory conditions the possibility of raising sex reversed males to maturity for breeding tests offered itself at once. But preoccupied with other work this project was delayed until the fall of 1953. Under the conditions of our laboratory sex reversed as well as normal animals reach sexual maturity shortly before they are one year old. Thus it has now become possible to present this report on offspring from three male parents.

Methods. The stock of Xenopus, originally obtained from Ciba-Basel, reproduces freely during summer months while kept outdoors in a concrete pond. In the laboratory, which is maintained at a constant temperature of 20°C, spawning occasionally occurs also spontaneously, but usually laying is induced by single

TABLE I. Effect of Estradiol on Sex Ratios in Xenopus (Total Losses and Mortality 26%).

Concentration, μg/liter	No. of ♂	No. of ♀	Total
1000	0	70	70
500	0	88	88
250	0	62	62
100	0	54	54
50	0	112	112
25	3	22	25
10	11	16	27
Controls	126	113	239

* Aided by a grant from the Rockefeller Foundation. The authors are also indebted to Ciba Pharmaceutical Products, Inc., for the generous supply of the steroid hormones and to Dr. Blanchard of Schieffelin and Co., New York, for the gift of the chorionic gonadotrophin.

TABLE II. Effect of Androgens on Sex Ratios in Xenopus (Total Losses and Mortality 28%).

Hormone	Conc., μg/liter	No. of ♂	No. of ♀	Total
Testosterone propionate	1000	38	30	68
Idem	100	48	30	78
"	10	24	34	58
Androsterone	1000	37	30	67
"	100	30	43	73
Controls		126	113	239

injections of 500 I.U. of chorionic gonadotrophin into the female, followed sometimes by a smaller dose given to the male. Steroid hormones are administered to larvae, usually starting at the time when they begin feeding, by injection of alcoholic solutions into the aquarium water. Tuberculin syringes are used to control exactly the amounts supplied and care is taken not to inject more than maximally .1 ml alcohol per liter of water. Control experiments have shown that this amount of alcohol exerts no detectable influence on either general growth or sexual differentiation of the larvae. Water and solutions are renewed 3 times per week.

Initial sex reversal. The effects of some steroids on sex differentiation of Xenopus larvae are presented in Tables I and II and summarized in Table III. It is evident that estrogens within a wide range of dosages prevent testicular differentiation in genetically male larvae. As a sequence to these experiments another 80 larvae were treated with 100 μg estradiol/liter and 30 larvae with 25 μg estradiol/liter of aquarium water. Hormone treatments were discontinued at metamorphosis in the first group and two months later in the second. Of the first group, 15 were later preserved and all were found to be of female morphology; several of the second group were laparotomized and showed the same result. The remaining 95 attained sexual maturity at the age of 11 months. None developed any external male sex characters, but all have enlarged cloacal papillae, a sure indication of circulating ovarian hormones.

TABLE III. Comparison of the Effects of Estrogens and of Androgens on Sex Differentiation in Xenopus.

Hormones	♂	♀	Total
Estradiol (50 μg-1000 μg/l)	0	386	386
Androgens (10 μg-1000 μg/l)	177	167	344
Controls	126	113	239

Breeding of sex reversed males. Statistically half of the 95 survivors of the last described estradiol groups may be expected to have been genetic males at the outset of the experiment. Limitations of space prevent submitting at once all 95 animals to the breeding test. So far only 8 have been used for this purpose, but the two principal questions have already found their answer. From the sex distribution among their offspring it can be concluded that (a) the *genetic sex* of the phenotypically reversed male remains *unchanged,* and (b) *the male is homozygous* in regard to sex determining genes. Sex reversed males bred with normal males produce, therefore, uniformly male offspring.

TABLE IV. Breeding of Estrogen-Treated Stock with Control Males.

Mother	Father	Offspring ♂	Offspring ♀
M_1	P_1	32	29
M_2	P_2	No offspring	
M_3	P_3	31	33
M_7	P_4	43	41
M_9	P_5	26	—
"	P_6	261	—
M_{10}	P_8	70	72
M_{11}	P_1	173	—
M_{12}	P_7	257	—
"	P_3	180	—

In detail, breeding followed the course indicated in Table IV. The second female that was chosen for testing, failed to deposit eggs even though stimulated with chorionic gonadotropin, and though males had embraced her in regular copulation. When laparotomized, it was found that no oviducts were present and the ovulated eggs were decaying in the body cavity. In a paper now being prepared by Segal, Chang, and Witschi, it will be shown that oviduct formation is inhibited if treatments with dosages of 50 μg or more are continued beyond metamorphosis. Obviously this one animal had developed a little ahead of others and the initial stage of oviduct

differentiation had come under the influence of the estradiol. The other 7 spawned successfully, 2 already twice in succession. The data on sex distribution among the offspring prove that of the egg producing mothers 4 were genetic females (nos. 1, 5, 7, 10) and 3 were genetic males (nos. 9, 11, 12).

Discussion. The effects of steroid hormones on sex differentiation present an interesting contrast to those obtained by heterosexual parabiosis and gonad transplantation in the same species(4). In the latter experiments the testis (medulla) severely inhibits the development of the ovary (cortex), and in some cases even leads to its testicular transformation. In the hormone experiment, the androgens do not interfere with ovarian development but cause a partial feminization of the testes(5). Since similar evidence was obtained also from experiments with toads, salamanders and birds, the notion that the sex hormones might be identical with the gene- or inductor-substances of embryologic sex differentiation is obviously inadequate. The mode of hormonal interference with the normal course of genetically controlled sex differentiation can, however, be clarified by the study of the primary reactions in the inductor systems of the differentiating sex glands. Cases analyzed thus far lead to the conclusion that the hormones act by differential inhibition of cortex or medulla(6-8).

There are now on record several breeding experiments with sex-reversed amphibians. In one instance, where reversal was incomplete, it was even possible to perform self-fertilization(9,10). These frogs, each one producing simultaneously mature eggs and sperms, and thereby an offspring of its own, are so far the only known doubly fertile hermaphrodites among tetrapod vertebrates. Most closely related to the present case are the ones reported by Ponse(11) for the toad Bufo and by Gallien(8) for the newt *Pleurodeles*. As in Xenopus, genetically homozygous males were successfully induced to produce mature eggs and to mate with normal males. Also in both instances entirely male offspring groups were obtained. Evidently, genetic sex constitution exerts no direct control over gametogenesis, somatic differentiation, or reproductive behavior. Its influence normally seems limited to the decision over cortical or medullary prevalence at the time of sex differentiation, that is, to a relatively short period of embryonic (larval) development.

Since among amphibians, and particularly anurans, Xenopus is best adapted to breeding under laboratory conditions, the described experiments gain a certain practical aspect. Sometimes it is of advantage to work with animals of one sex only and to know whether they shall be males or females, even at an early stage. With a few sex-reversed males at hand, it is an easy matter to produce exclusively male offspring by the hundreds or thousands. Moreover, any desired number of females may be derived from such all-male cultures by the addition of some estrogenic substance to the aquarium water, during the larval stage. Hence it will now be an easy matter to propagate exclusively and indefinitely the genetically male type Xenopus, with complete elimination of the heterozygous female type—if so desired.

Summary. Xenopus larvae of male genetic sex develop into phenotypic females, if raised in weak solutions of estradiol (25 to 1000 μg/l). Such feminized males, if bred to normal males, produce uniformly male offspring. It is concluded that in Xenopus the males are the homozygous sex and that phenotypic sex reversal does not affect the male genetic constitution. The practicability of breeding entirely homozygous male and female laboratory stock of Xenopus is pointed out.

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Enzymatic Degradation of the Side-Chain of Cortisone: Conditions for an Enzyme Assay.* (21743)

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This report describes an a-cellular enzyme system in rat liver which degrades the 17, 21-dihydroxy-20-ketone side-chain of adrenocortical steroids. The product or products of the reaction have not yet been identified.

Methods. All animals used in these studies were male rats (Holtzmann Rat Co., Madison) fed *ad libitum;* weights varied from 150 to 300 g. All homogenizations were carried out at 0°C in a tight-fitting all-lucite homogenizer of the Potter-Elvehjem type. The homogenization medium employed changed with the progress of the work. The reaction was carried out in test tubes equipped with side arms. Reaction mixtures were extracted in 5 volumes of chloroform. The chloroform layer was washed once with ¼ volume of 0.1 *N* NaOH, once with ¼ volume of 0.1 *N* HCl, and once with ¼ volume of water. An aliquot of the washed chloroform layer was dried down and the residue taken up in methanol. The sulfuric acid-phenylhydrazine reaction of Porter and Silber(2) was used to follow degradative alterations of the 17, 21-dihydroxy-20-ketone side-chain. Tissue blanks were invariably extremely low, which indicated absence of interfering materials. Color intensities were estimated in the Coleman Jr. spectrophotometer at 410 mμ. Analyses in the Beckman spectrophotometer at 370, 410 and 450 mμ indicated that the Allen correction(3) was unnecessary.

Results. Preliminary experiments indicated that significant losses of the 17, 21-dihydroxy-20-ketone side-chain of cortisone were induced by whole homogenates of rat liver if anaerobic conditions were maintained. The rate of the reaction was markedly increased in the presence of relatively high concentrations of diphosphopyridine nucleotide (Fig. 1), which clearly implicated pyridine nucleotides in the reaction. However, if the homogenization of the liver was carried out in 0.25 *M* sucrose, made 0.04 *M* with respect to nicotinamide, no effect of added DPN could be demonstrated. Later work(4) suggested that reduced triphosphopyridine nucleotide (TPNH) is the active cofactor in the reaction. On the basis of the data of Fig. 1, the homogenization medium routinely employed (except where noted) was 0.25 *M* sucrose,

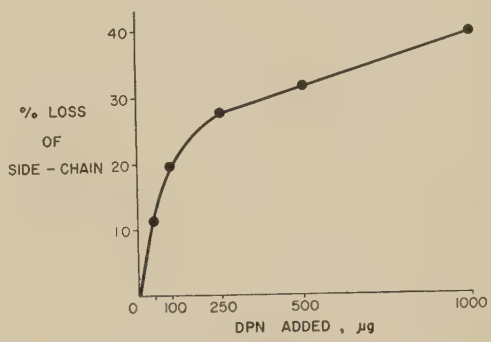

FIG. 1. Effect of diphosphopyridine nucleotide. Conditions: 250 mg of whole rat liver homogenized in 0.25 *M* sucrose; 100 μM potassium phosphate buffer, pH 7.4, 180 μg cortisone, final volume 4.5 ml, anaerobic conditions, 1 hr incubation at 37°C. Loss of the side-chain is plotted as % of added cortisone.

TABLE I. Saturation Level of Added Cortisone.

Cortisone added, μg/5 ml of reaction mixture	Cortisone lost, μg
36	35
72	36
108	55
144	58
180	60
216	54
252	59
288	59
324	64

Conditions: 200 mg whole rat liver homogenized in 0.15 *M* KCl, 200 μM potassium phosphate buffer, pH 7.4, 500 μg DPN, final vol., 5 ml, anaerobic conditions, 2.5 hr incubation at 37°C.

* This investigation was supported by research grants A-329(C2) and A-331(C2) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service. A preliminary report has been published(1).

† Data presented in this report are in partial fulfillment of the requirements for the Ph.D. degree.

made 0.04 *M* with respect to nicotinamide.

Ethylenediamine-tetra-acetic acid (Versene) was added in a few instances to a final concentration of 0.001 *M*. No consistent differences with or without Versene could be demonstrated. The enzyme system was found to be saturated at a final concentration of 25 μg cortisone per ml of reaction mixture (Table I). The enzyme system has a sharp optimum in the region pH 7.4 to 8.0 (Fig. 2). A study of the effect of temperature on the reaction revealed a typical increase in rate which leveled off at an optimum temperature of 38° to 40°C, and fell off sharply at higher temperatures. Homogenization in distilled water resulted in a 42% loss of activity as compared with homogenization in 0.25 *M* sucrose, or isotonic sodium or potassium chloride.

On the basis of these experiments the final conditions were as follows: Homogenization was carried out in 0.25 *M* sucrose made 0.04 *M* with respect to nicotinamide. The reaction mixture, adjusted to a final volume of 5.0 ml, contained 180 μg cortisone, 100 μM potassium phosphate buffer (pH 7.4) and 10 μM nicotinamide. Sucrose was added to give a final osmotic concentration of all constituents equal to 0.25 osmolar. Incubation was carried out anaerobically at 38°C.

Survey of various tissues for the enzyme system. Sucrose-nicotinamide homogenates were prepared from rat liver, kidney, skeletal muscle, gastrointestinal tract (including pancreas), brain and lungs. Assays for alteration of the side-chain of cortisone were carried out in the system as described above (Fig. 3).

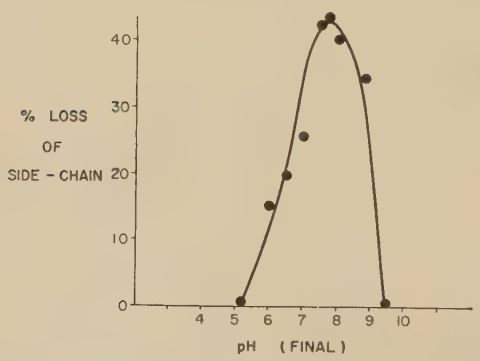

FIG. 2. pH optimum. Conditions: 250 mg whole rat liver homogenized in 0.15 *M* KCl, 360 μg cortisone, K_2HPO_4-citrate buffer, 2 hr incubation at 37°C, other conditions as in Table I. Loss of the side-chain is plotted as % of added cortisone.

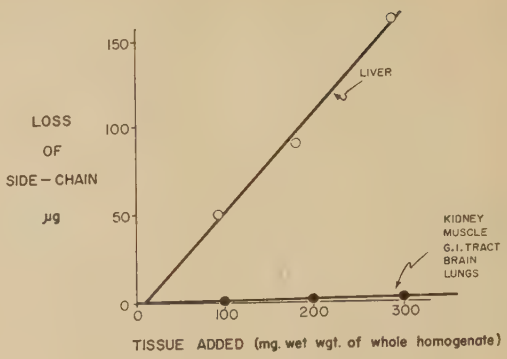

FIG. 3. Tissue survey.

The strict linearity of the activity-enzyme concentration curve for the liver homogenate may be noted. In a large number of later experiments(4) using the above conditions, the enzyme activity as evidenced by alteration of the cortisone side-chain was not consistently linear with respect to the amount of added homogenate, or fraction derived therefrom. However, this effect was observed only at relatively low concentrations of whole homogenate, or in partially purified systems. This phenomenon is the so-called "dilution effect" observed at lower enzyme concentrations, *i.e.*, the decrease in activity of the enzyme is more than proportional to the dilution factor alone. This effect is typical of multi-enzyme systems in which some cofactor is being supplied by an auxiliary system. The "dilution effect" proved an important clue leading to the possible cofactor involved(4).

Instability of the Enzyme System. The data plotted in Fig. 4 and Table II indicate the instability of the enzyme system. For these experiments 5% whole homogenates of rat liver were prepared in 0.25 *M* sucrose which was

TABLE II. Instability on Handling.

Preparation	Cortisone lost, μg
Original homogenate	180
Same, after 1st sedimentation and resuspension	100
Same, after 2nd sedimentation and resuspension	50
Same, after 3rd sedimentation and resuspension	15

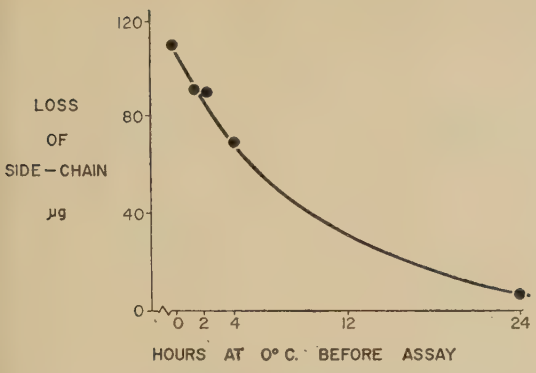

FIG. 4. Instability on standing at 0°C. Conditions: 100 mg whole rat liver homogenate, 2.5 hr incubation at 37°C; other conditions as for the standard assay as described in the text.

0.04 *M* with respect to nicotinamide and 0.001 *M* with respect to Versene. For the data plotted in Fig. 4, the whole homogenate was stored at 0°C and assayed at intervals as shown. As may be seen, the activity declined steadily and was almost completely lost after 24 hours.

Losses of the enzyme system were greatly increased on handling (Table II). In this experiment an aliquot of the original homogenate was assayed immediately following homogenization. The remainder was centrifuged at 11,000 x gravity for 60 min. The particulate fraction was then resuspended in the supernatant fraction and an aliquot removed for assay. This procedure was repeated twice. The total elapsed time from the preparation of the original homogenate to the completion of the third sedimentation and resuspension was 4 hours. As the data show, repeated handling greatly increased the decay of the enzyme system.

Discussion. Pregnanediol is a metabolite of progesterone(5). That a significant fraction of the metabolism of the adrenocortical steroids also involves a reduction of the C-20 ketone to the corresponding hydroxyl group has long been suspected. Four new metabolites of adrenocortical steroids have recently been isolated from human urine(6). The distinguishing feature of these compounds is the presence of a 17, 20, 21-triglycol side-chain. Therefore, the C-21 steroids of the progesterone and adrenocortical steroid groups appear to share a common pathway insofar as reduction of the C-20 ketone is concerned. The loss of Porter-Silber reacting material (*i.e.*, cortisone) under reductive conditions as reported in this paper, suggests that the enzyme system described is the one responsible for the reduction of the C-20 ketone. Resolution of this question awaits the requisite paper chromatographic studies which are being carried out at present. Further studies on the intracellular localization of the enzyme system are reported in the following communication.

Summary. An enzyme system is described which degrades the 17, 21-dihydroxy-20-ketone side-chain of adrenocortical steroids. The enzyme system requires added nicotinamide, anaerobiosis, and isotonic conditions for optimum activity. The enzyme system was found only in the liver.

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Enzymatic Degradation of Side-Chain of Cortisone: Intracellular Localization of the Enzyme System.* (21744)

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In the first paper in this series(2) an enzyme system in rat liver is described which degrades the 17,21-dihydroxy-20-ketone side-chain of cortisone. In this report studies on the intracellular localization of the enzyme and its specificity are presented.

Methods. Cell fractionation was conducted at 0°C according to Schneider(3) with modifications as indicated. The homogenization medium employed was 0.25 *M* sucrose containing 0.04 *M* nicotinamide and 0.001 *M* Versene. Enzyme activity was assayed using the final conditions as previously described(2), although certain modifications were occasionally introduced with no effect on the rate or character of the reaction. Tissue extraction and steroid analysis for the 17,21-dihydroxy-20-ketone side-chain were carried out as described in the previous paper(2). Reduction of the Δ^4-3-ketone was followed in the Beckman spectrophotometer at 240 mμ. Tissue blanks gave insignificant readings.

Results. The nuclei were sedimented at 600 $\times$ gravity and washed once. The activities of the nuclear fraction and the nuclei-free homogenate were assayed and compared with that of the unfractionated whole homogenates (Fig. 1). In Fig. 1 and subsequent figures the term equivalent milligrams refers to the milligrams wet weight of whole liver which would give rise, on fractionation, to the quantity of cell fraction actually used in the experiment. The complete absence of activity in the isolated nuclei, as well as the quantitative recovery of the enzyme system in the nuclei-free homogenate is evident.

In experiments on mitochondria the following procedure was adopted. The whole homogenate was cleared of the nuclear fraction by centrifugation at 600 $\times$ gravity. The nuclear sediment was washed free of mitochondria and discarded. The combined supernatant was centrifuged at 6000 $\times$ gravity for 15 minutes. The mixed mitochondrial and microsomal residue was resuspended and recentrifuged, the supernatant and loosely sedimented layer, *i.e.*, the so-called "fluffy layer"(4) was removed and added to the original supernatant fraction. The activities of the washed mitochondria and the final nuclei- and mitochondria-free supernatant were compared with the nuclei-free homogenate. This procedure is valid if the nuclei are inert, as has been shown. The results of one experiment are shown in Fig. 2. The low order of activity in the mitochondrial

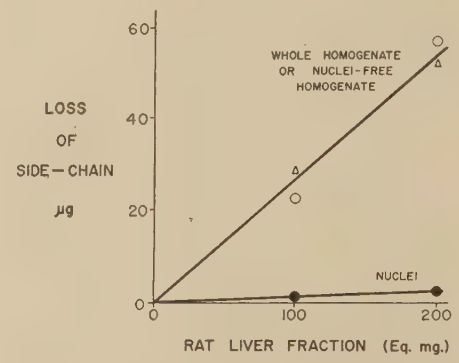

FIG. 1. Inactivity of nuclear fraction.

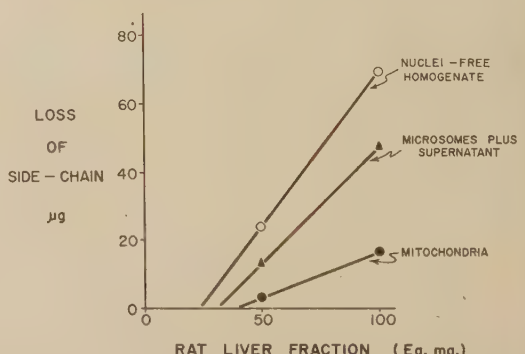

FIG. 2. Distribution of activity between mitochondria and supernatant.

* This investigation was supported by research grants A-329 (C2) and A-331 (C2) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health. A preliminary report has been submitted(1).

† Data presented in this report are in partial fulfillment of the requirements for the Ph.D. degree.

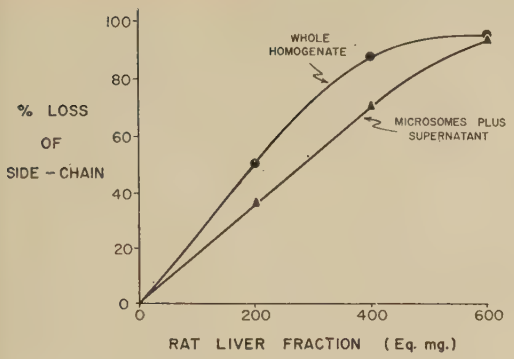

FIG. 3. Recovery of enzyme system in microsome-supernatant fraction.

fraction, as well as the marked displacement to the right (dilution effect) of the activity-enzyme concentration curves may be noted. In a repetition of this experiment the mitochondrial fraction showed zero activity.

Demonstration that the nuclei and mitochondria were inactive simplified the preparation of highly active liver extracts. The procedure adopted was as follows: The whole homogenate was centrifuged at 6000 × gravity to remove nuclei and mitochondria in one step. The residue was resuspended and washed once, any loosely packed material removed and added to the supernatant fraction. This procedure led to recoveries in the microsome plus supernatant fraction of 70% of the activity of the unfractionated whole homogenate (Fig. 3).

Initial attempts to localize the enzyme in either the microsome or final supernatant fractions were unsuccessful because of the instability(2) of the enzyme system. Fig. 4 shows data from an experiment in which the sum of the activities of the microsomes plus supernatant were far less than the activities of the two fractions when acting together. The marked dilution effect may be noted. In other experiments the activities of both the microsomal and supernatant fractions completely disappeared on separation. The activity of the isolated microsomes was unaffected by addition of either oxidized or reduced diphosphopyridine nucleotide (Sigma Chemical Co.) or by addition of boiled supernatant. However, that reduced triphosphopyridine nucleotide (TPNH) was the active cofactor in the reaction was suggested when the "dilution effect" was abolished by addition to the microsome plus supernatant fraction of citrate plus magnesium (Fig. 5). Marked augmentation of the reaction was also observed following addition of isocitrate or glucose-6-phosphate. Since the oxidation of isocitrate and glucose-6-phosphate both proceed through triphosphopyridine nucleotide, this cofactor is implicated in the reaction.

Data which show that the enzyme system responsible for alteration of the side-chain is present in the microsome fraction are presented in Table I. An homogenate free of nuclei and mitochondria was prepared as described. A portion of this preparation was centrifuged at 20,000 × gravity (maximum gravitational field available) for 60 minutes. The resulting microsomal pellet and residual supernatant were assayed for side-chain alteration and reduction of the Δ^4-3-ketone grouping. The enzyme system responsible for reduction in ring A is localized in the super-

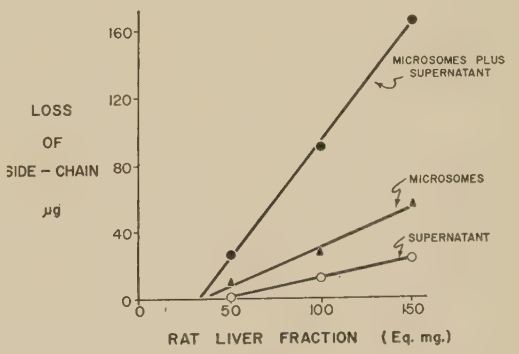

FIG. 4. Loss of activity on separation of microsomes.

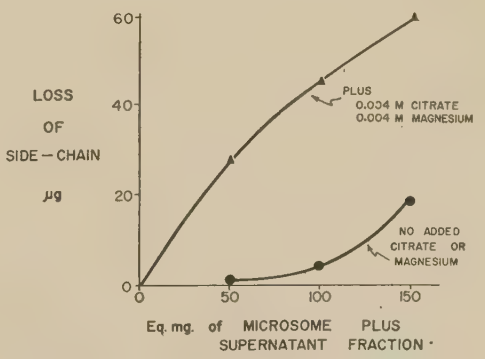

FIG. 5. Abolition of dilution effect.

TABLE I. Effect of Isocitrate and Glucose-6-Phosphate on Cortisone Degradation, and Partial Separation of the System Degrading the Side-Chain from the System which Reduces Ring A.

	Disappearance of cortisone	
	Loss of side-chain (phenylhydrazine), μg	Loss of Δ^4-3-ketone (U-V absorption), μg
100 eq. mg microsomes + supernatant,		
+ .004 *M* isocitrate	53	132
+ .003 *M* glucose-6-phosphate	63	110
100 eq. mg microsomes alone,		
+ .004 *M* isocitrate	32	51
+ .003 *M* glucose-6-phosphate	17	4
100 eq. mg supernatant alone,		
+ .004 *M* isocitrate	28	122
+ .003 *M* glucose-6-phosphate	23	120

natant fraction, in confirmation of Tomkins and Isselbacher(5). The enzyme system carrying out the alteration of the side-chain is present to the extent of approximately 50% in the microsome fraction. The question as to whether the enzyme system is associated with the smaller electron dense particles(6) known to sediment only in gravitational fields in the neighborhood of 100,000 × gravity(7), or whether the microsome and supernatant fractions contain different systems, each able to carry out an alteration of the side-chain, must await further work.

The data of Table II show the loss of a number of different steroids in a 90 minute incubation under the standard conditions of the assay. The enzyme preparation used was a nuclei and mitochondria-free homogenate of rat liver.

Discussion. The recent experiments of Fukushima *et al.*(8), in which urinary products of cortisone reduced at C-20 were obtained in high yield, indicate that reduction of the characteristic 17,21-dihydroxy-20-ketone configuration to the glycol may be a major metabolic pathway of the adrenocortical steroids. This hypothesis is at variance with conclusions reached by Hechter *et al.*(9), which suggested that cortisone was not being converted to the 17,20,21-triglycol in perfusion studies, although later work(10,11) indicated some formation of the reduced product. The experiments presented in this and the preceding paper suggest that the disappearance of Porter-Silber reacting material involves a reduction at C-20. If this should prove to be the case, then the *in vitro* experiments reported here may be considered to directly supplement the *in vivo* experiments of Fukushima *et al.*(8). Speculations(10,11) on the question of the sequence of reactions followed in the metabolism of adrenocortical steroids in the liver have been restricted to inferences derived from a study of metabolic products in tissue slice and whole organ perfusion studies. The present experiments are of interest in regard to this question, since this problem would be greatly facilitated if the enzymes catalyzing single step reactions could be isolated and their specificities determined.

TABLE II. Loss of Side-Chain of Different Steroids.

Steroid added	Loss of steroid side-chain, μg
Hydrocortisone	65
Δ^4-Pregnene-17 α, 21-diol-3,20-dione	85
Cortisone	108
Tetrahydrocortisone	115

From the point of view of the biochemistry of intracellular particulates, it may be pointed out that the data presented is suggestive of a localization of the enzyme in the microsome fraction, but does not show that the enzyme is present partially in solution. A revised assay system, employing addition of an appropriate TPN-linked dehydrogenase, is being used in the further investigation of this problem. The present data have been presented primarily to draw attention to the nature of the enzyme system and to give a provisional statement as to the intracellular localization.

Summary. The intracellular localization of an enzyme system which alters the 17,21-dihydroxy-20-ketone side-chain of cortisone was studied in rat liver. The nuclei and mitochondria were inactive. The enzyme was found to be approximately equally distributed between a microsome fraction sedimented at 20,000 × gravity and the supernatant fraction. The enzyme activity was greatly enhanced on addition of magnesium and either glucose-6-phosphate, citrate, or isocitrate.

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Non-Esterified Higher Fatty Acids in Serum of CCl_4-Treated and Normal Rats and Other Species. (21745)

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Studies on the nature of the azorubin-binding capacity (ABC) of serum albumin suggested that the lowering of the ABC values observed in certain "pathological" sera was caused by increased concentrations of anions bound firmly to albumin. This assumption was supported by model studies in which the ABC of serum albumin decreased after addition of higher fatty acids(1), and by the finding that the electrophoretic mobility of albumin in low-ABC sera indicated a higher amount of firmly bound anions(2).

Inasmuch as this evidence was of an indirect nature, proof was sought by a direct analysis of sera which contained low ABC albumin. The concentration of "free," *i.e.* non-esterified higher fatty acids* was determined in the sera of rats in which the ABC was reduced by treatment of the animals with carbon tetrachloride(3); the resulting values were compared with those in normal rat sera. In addition, albumin was isolated from the serum of normal and carbon-tetrachloride-treated rats and the content of higher fatty acids estimated. Normal bovine and rabbit sera were included in studies on the reproducibility of the procedure.

Materials. 1. *Reagents.* All organic solvents employed were redistilled before use. Stearic acid (c.p.) was obtained from Fisher Scientific Co. All other chemicals were reagent grade.

2. *Sera.* The sera of the rats and rabbits were obtained as outlined before(3,4) after a 24 hr fast. The analytical samples were taken from 4 pools each of the normal and CCl_4-rat sera, prepared from a total of 144 and 155 animals, respectively. The rabbit serum was pooled from 9 animals. The sera were deep-frozen at once after preparation, and thawed immediately before extraction.

* The term "free higher fatty acids", generally used in the literature for non-esterified higher fatty acids in serum, is inaccurate since these acids, in physiological concentrations, are known to be firmly bound to albumin.

The temperature during thawing was kept as low as possible. Four lots of bovine sera were used in the analyses; they were prepared as soon as possible after obtaining the blood from the slaughter-house, and deep frozen.

3. *Albumin preparations.* Albumin was prepared by a slight modification of the ammonium sulfate precipitation used by Kendall (5). The fraction between 60 and 95% saturation was collected, dialyzed at 3°C against water until free of sulfate ions, and lyophilized. Electrophoretic analyses (pH 7.7, phosphate buffer) of the albumin preparations obtained from normal and carbon tetrachloride-treated rats failed to demonstrate protein components other than albumin.

Extraction. 1. *Sera.* The serum sample (15 ml) was added to a mixture of 10 ml of a 1% sodium chloride solution and 4 ml 1 N hydrochloric acid. The solution was extracted once with 8 ml and then 6 times with 4 ml of ether, using a glass plunger(6) for gentle mixing. The ether phase was separated by centrifugation for 10 min. at approximately 15°C and 800 g. The combined ether extracts were washed with 5 ml portions of a 1% sodium chloride solution to litmus-neutrality of the aqueous phase. The ether solution was then transferred into the titration vessel where the solvent was evaporated in a warm water bath under a stream of nitrogen. Overheating was prevented by a few grains of 40 mesh sand which had been washed with acid, base, water, alcohol and ether.

2. *Albumin Preparations.* The lyophilized albumin was pre-extracted with dry ether in a Soxhlet extractor for 24 hr and liberated from adhering ether in a vacuum desiccator over paraffin shavings. After this treatment, the albumin preparation from the carbon tetrachloride-treated rats showed an ABC value 21% lower than that from normal rats(3). The albumin was then dissolved in water, denatured by boiling for several minutes and extracted by refluxing with a 3:1 alcohol-ether mixture for 45 min. The extract was concentrated on the water bath with a stream of nitrogen and then distributed between petroleum ether and 0.2 N hydrochloric acid. The residue of the washed lipid phase was titrated as described below.

Titration. The conical titration vessel (6 ml) ended in a male 24/40 ST joint which was fastened to a female ST joint "holder." The holder accommodated 2 stainless steel needles: (a) the buret tip and (b) a nitrogen delivery tube which extended to the bottom of the vessel. The nitrogen was passed through a sodium hydroxide solution and water containing phenolphthalein. The system had a pressure-release tube between the wash columns and the delivery needle.

The *residue* of the ether extract was dissolved in 0.5 ml ethanol, 1 drop of 0.1% Nile blue in ethanol was added and nitrogen was bubbled through for 10 minutes. The solution was titrated with aqueous 0.02 N sodium hydroxide to a just-pink endpoint. A 1% sodium chloride solution replaced the serum in the blank experiments. Larger volumes were employed in the titration of the acid material obtained from the albumin preparations.

Recovery Experiments. Stearic acid was selected as a model of the higher fatty acids which were to be recovered by the present procedure. The efficiency of the titration and extraction methods was checked in experiments in which 2-28 microequivalents of stearic acid were used. Direct titration of alcoholic solutions of the acid (36 determinations) gave an average recovery of 102% (S. E. $= \pm 0.77\%$). In analyses of mixtures of stearic acid or sodium stearate and 1% sodium chloride (10 determinations) by the above procedure, an average of 103% (S.E. $= \pm 1.52\%$) was recovered. Application of the technic to a solution of stearic acid in bovine serum (11 determinations) resulted in an average recovery of 100% (S.E. $= \pm 1.96\%$). The solution was prepared by gently shaking bovine serum at 3°C in a vessel containing a thin film of stearic acid or sodium stearate. The recovery value was calculated as the difference between simultaneously determined samples with and without stearic acid.

Discussion of method. The present procedure determines the ether-soluble, water insoluble fraction of the acids present in the serum. These are essentially the higher fatty acids which are known to be firmly bound to the serum albumin. The recoveries obtained are considered satisfactory for the purpose of

TABLE I. Non-Esterified Higher Fatty Acids in Various Sera.

Units	Normal rats	CCl_4-treated rats	Normal rabbits	Bovine serum
No. of determinations	11	7	5	29
Microequivalents/ml ± S.E.	.51 ± .023	.61 ± .029	.52 ± .020	.43 ± .017
Albumin, g %*	4.13(3,8)	3.25(3)	3.62(4)	3.58
Moles F.A.†/mole albumin ± S.E.	.85 ± .039	1.30 ± .062	.99 ± .039	.83 ± .033
Fiducial limits (0.99 prob.) ±	.12	.23	.18	.09
Moles F.A.†/mole albumin‡	.91	1.20		

* Figure in parenthesis gives reference in bibliography.
† F.A. = Non-esterified higher fatty acids; moles calculated as mono-carboxylic acid.
‡ Values obtained in analysis of albumin preparations.

the present investigation. The titration error did not exceed 0.005 ml 0.02 N sodium hydroxide.

The sera were adjusted to pH 2 in order to achieve highest efficiency of the extractions. This was preferred to the extraction at pH 6 in the presence of comparatively large quantities of sodium dodecyl sulfate(6). Nitric acid which binds more firmly to albumin than other mineral acids(7) and which was therefore expected to facilitate the extraction of the albumin-bound fatty acids by effective displacement, caused some turbidity not encountered in the use of hydrochloric acid. Diethyl ether proved preferable to petroleum ether (6) as an extractant since it presented less difficulty with gel formation.

Results and discussion. Table I gives the results obtained in the analysis of the sera and albumin preparations. The concentration of non-esterified higher fatty acids found in normal rat serum is in accordance with that reported by Grossman and associates(9). Kelsey and Longenecker(10) found in beef plasma a concentration of "free" fatty acids approximately twice that given in Table I for bovine serum; they point out, however, that their value may be high because of possible lipolysis. It may be noted that albumin in the sera of the three species analyzed as well as the albumin preparation from normal rat serum, contained slightly less than one mole of higher fatty acids per mole albumin,† a value similar to that found for human serum albumin(11). The higher amounts of fatty acids observed by Kendall in crystalline human albumin preparations may be indicative of lipolytic processes in the plasma used as starting material which had been stored, presumably in the liquid state, for periods up to 6 months(5). On the basis of the studies by Grossman and associates on lipolysis in rat serum(9), the values obtained in the present studies are believed not to be influenced by hydrolytic processes.

The elevated ratios "moles of non-esterified higher fatty acids per mole albumin" found in the material from carbon tetrachloride-treated rats (Table I) are considered further evidence for the previous assumption that the lowering of the ABC of albumin in certain "pathological" sera is caused by increased concentrations of anions bound firmly to albumin(1). The results are also in accordance with the higher electronegative charge of albumin in low-ABC sera(2).

It has been concluded(1,2) that the albumin in the low ABC serum (carbon tetrachloride-treated rat) contained approximately one more electro-negative charge than that of normal serum. Since only about half this amount was found as an average value in the present investigation, it should be pointed out that the determination of the extremely small quantities of non-esterified higher fatty acids present in serum is subject to a comparatively wide range of error, as indicated by the fiducial limits given in Table I. On the other hand, it has been emphasized(2) that the net charge difference observed for the two types of albumin has no greater accuracy than that of an order of magnitude. In view of these methodological limitations, the analytical data of the present study are considered to be in

† A molecular weight of 69000 was used for albumin of all sera in order to maintain a comparison between the species, and with values reported for human serum albumin(11).

satisfactory agreement with the previous conclusions on the increased anion content of the low-ABC albumin.

Preliminary experiments(12) have shown that the "higher fatty acid fraction" from normal and carbon tetrachloride-treated rats contained at least two components which could be separated by paper chromatography(13); their migration rates corresponded to those of stearic and palmitic acid, respectively.

The increased ratio "higher fatty acids per mole albumin" results from two metabolic aberrations, *i.e.*, elevated level of higher fatty acids and decreased albumin concentration. Reduced utilization of higher fatty acids in the carbon tetrachloride-treated rat has been observed by Winter(14). Low albumin values have been found in the same experimental animal(3) as well as in other cases of liver injury. In reference to the low ABC values found in tourniquet-shock rats for which a similar explanation was postulated(1,2), it should be pointed out that the liver function is also known to be impaired in this condition (15). Reduced oxidation of fatty acids by the liver(16) and decreased serum albumin levels have been observed in shock(3,4).

Serum albumin with low ABC values has been demonstrated in various clinical cases (17). Zirm and Axenfeld(18) observed recently in similar pathological conditions an impaired ability of albumin to bind oleic acid. It is of interest that most of the patients with low-ABC albumin have been found deficient in their serum albumin levels; in many of them, clear evidence of liver damage was available(17). On the basis of the results obtained in experimental animals it is suggested that the clinical sera with decreased binding capacities of albumin for azorubin or oleic acid are characterized by abnormally high amounts of higher fatty acids bound per mole albumin.

Summary. 1. Available methods for the determination of the "free," *i.e.* non-esterified, higher fatty acid fraction in serum were modified. These acids are firmly bound to albumin. Slightly less than one mole of them was found per mole albumin in normal rat, rabbit and bovine serum. 2. Serum of carbon tetrachloride-treated rats contained more non-esterified higher fatty acids (1.30 moles per mole albumin) than that of normal rats (0.85 mole per mole albumin). The difference was statistically significant. 3. The results are considered indicative of the impaired liver function in the injured rats. They offer further evidence for the postulation that the lowering of the azorubin-binding capacity of serum albumin is caused by increased concentrations of firmly bound higher fatty acids.

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Abortion, Stillbirth, Early Death of Young in Rabbits by *Listeria monocytogenes*. I. Ocular Instillation.*† (21746)

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Listeria monocytogenes is associated most commonly with an acute, highly fatal encephalitis of ruminants. Recently, however, attention has been focused on the role of this bacterium in widespread outbreaks of abortion, stillbirth, and early death of infants (*granulomatosis infantiseptica*) in the human population of both sectors of Germany(1-3, and others). A similar condition has been observed in foals(4,5), lambs(6,7), and calves (8-11). Gray(12) cited 9 reports of abortion in cattle, 5 in sheep, and one in rabbits. This suggests that abortion or early death due to *L. monocytogenes* actually may be widespread but unrecognized in both human and animal populations. In a study of the possible mode of infection leading to abortion in pregnant laboratory rabbits, it was found that abortion, stillbirth, and early death of young could be produced consistently both by ocular instillation of *L. monocytogenes* and also by the addition of suspensions of the specific bacterium to the drinking water(13).

It is well established that cultures of *L. monocytogenes* instilled into the eye of the rabbit or guinea pig produce a marked, "local", purulent conjunctivitis and keratitis. This observation was reported almost simultaneously by Anton(14), and Morris and Julianelle(15). The specific nature of the reaction led Julianelle and Pons(16) to suggest this as a rapid means of identifying suspect cultures of the bacterium. During the past 2 decades this technic has been accepted as a standard procedure in the identification of *L. monocytogenes*. Most investigators employing this technic emphasized the local nature of the reaction. Rarely was mention made of general infection following ocular instillation. However, Graham *et al.*(17) and Gray *et al.*(18) each reported death due to listeric encephalitis of a pig and rabbit respectively, following ocular instillation. Asahi and Hosoda(19) reported successful production of encephalitic symptoms in goats following ocular exposure. This could not be confirmed by Hirato *et al.* (20) or in this laboratory. Anton(14) observed death due to listeric septicemia in a rabbit one week pregnant, and abortion in another rabbit late in gestation, following ocular instillation. Belin(21) instilled *L. monocytogenes* into the conjunctival sac of a pregnant guinea pig and it aborted several days later. At the time no particular significance was attached to these findings.

Materials and methods. Thirty-two female rabbits ranging in age from 8 to 18 months were exposed to *L. monocytogenes* by instilling 2 drops of culture into the left eye. Two different cultures were used. One was isolated from the medulla oblongata of a calf which died of listeric encephalitis complicated by vit. E deficiency, and the other was isolated by Potel(2) from the liver of an aborted infant. The cultures were grown at 37° C on Difco tryptose agar slants for 18 to 24 hours. They were suspended in sterile distilled water to a density approximating the number 8 tube of the McFarland Nephelometer when compared in a Cenco-Sheard-Sanford Photelometer, and instilled into the conjunctival sac by means of a capillary pipette. Thirteen rabbits were exposed at the end of 2 weeks gestation, 7 at 4 days or less prepartum and 12 were nonpregnant controls. Differential leukocyte counts and swab cultures from the left eye and vagina were taken each 3rd day after exposure for a period of 2 weeks. All aborted fetuses, and all young born at term which were stillborn or died shortly after birth, were necropsied and the liver and stomach contents of each were cultured. Urine was cultured when available. Five infected

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TABLE I. Number of Positive Cultures from Does, Fetuses, and Membranes.

	Liver	Spleen	Blood	Kidney	Uterus	Vagina	Urine	Eye
Abortion								
Aborted and died	2/2*	2/2	1/2	1/2	1/2	1/2	1/2	1/2
" " sacrificed	2/3	2/3	0/2	0/2	2/3	3/3	2/3	3/3
Died before abortion	1/2	2/2	1/2	1/2	2/2	2/2	0/2	2/2
Sacrificed before abortion	1/1	1/1	0/1	0/1	1/1	1/1	1/1	1/1
Aborted and died, 2nd exposure	1/2	2/2	1/2	1/2	2/2	2/2	2/2	2/2

	Liver	Stomach contents	Amniotic fluid	Urine	Cotyledons
Fetuses					
Aborted	47/55	41/55	5/5	—	12/12
Born at term	42/58	28/58	1/1	5/5	19/19

* Positive cultures/total specimens cultured.

does and 8 controls were sacrificed for necropsy. Liver, spleen, heart blood, kidney, uterus, urine, vagina, eye, and amniotic fluid and cotyledons when present, of all rabbits which died or were sacrificed were cultured. Cultures were made on Difco tryptose agar plates, incubated at 37°C for 18-24 hours after which they were examined by means of a binocular scanning microscope and oblique lighting (22, 18). Three does which aborted were rebred 8 weeks postabortion and reexposed during the 3rd week of gestation. Two were rebred 6 weeks postabortion but not exposed. Of the 7 does exposed near term, 2 were rebred 7 days after parturition, one at 17 days, one at 24 days, and one at 8 weeks. None were reexposed. All does which survived the 2nd gestation were rebred from 7 days to 6 weeks postpartum but not reexposed.

Results. Abortion. There was no significant difference in the reaction of the does exposed to the 2 different cultures. Of the 13 does exposed to *L. monocytogenes* by ocular instillation during the 3rd week of gestation 10 aborted within 96 hours. Two aborted as early as 24 hours after exposure. In general does which aborted early (within 48 hours) showed no clinical indication of illness, while most does which aborted between 48 and 96 hours definitely were ill and 2 died with listeric septicemia within 3 days after abortion. Three does failed to abort. However, one of these died with listeric septicemia on the 5th and another died on the 7th postexposure day. The 3rd doe was comatose on the 9th postexposure day and was sacrificed. The uteri of all 3 were necrotic, filled with pus, and contained from 7 to 9 fetuses. Many of these were macerated, partially liquefied, and *L. monocytogenes* was readily cultivated from them. Some does, either prior to or for several days following abortion, showed a marked vaginal discharge of thick, blood tinged mucous-like material which contained large numbers of the specific bacterium as determined by culture.

The 4 does which died, and the 4 which were sacrificed showed varying degrees of metritis at necropsy. This ranged from marked congestion to extensive necrosis. The uterus of does in which one or more fetuses or cotyledons were retained was usually distended and completely filled with thick creamy yellowish white pus which contained *L. monocytogenes*. Fetuses in these pus filled uteri always showed evidence of extensive resorption. In most instances the liver was mottled and pale yellow in color, but rarely showed focal necrosis. At times the kidneys were pitted and showed numerous petechiae. Gross lesions could not be detected in other organs. The number of positive cultures from the viscera is shown in Table I. The aborted fetuses ranged from well developed to badly macerated masses which often were difficult to identify (Fig. 1). In some instances when the conceptus was expelled intact the amniotic fluid was blood tinged and dark red in color, while in others it was normal in color but contained large flecks of yellowish material resembling pus. The cotyledons often were hemorrhagic or necrotic. *L. monocytogenes* was isolated in large

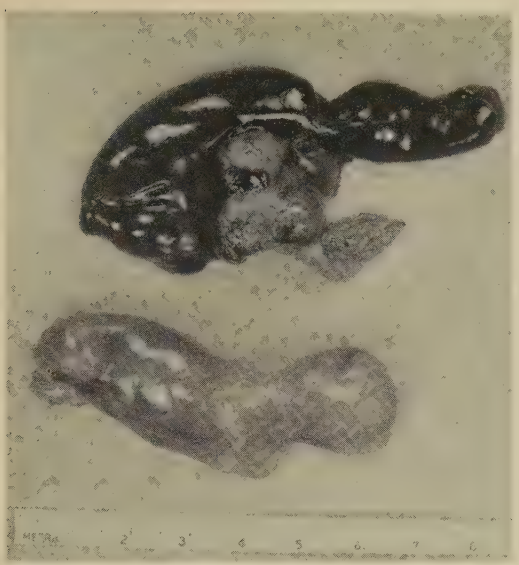

FIG. 1. Approximately 18-day-old litter mate fetuses aborted 4 days after exposure. Upper: necrotic cotyledon and blood stained amniotic fluid which contained a mummified fetus. Lower: well developed and normal appearing fetus. *L. monocytogenes* isolated from each.

numbers from the fluid and cotyledons. Detection of gross lesions in the viscera of the fetuses was difficult. In a few instances focal hepatic necrosis was observed, but no other lesions. The number of positive cultures from fetuses and membranes is shown in Table I.

Rebreeding. Two of the 3 does rebred 8 weeks postabortion and reexposed during the 3rd week of gestation died 5 days after exposure. Necropsy and cultural findings were similar to those described following initial exposure (Table I). The 3rd gave birth to a normal litter of 12, 2 of which died within the 1st week. No significant lesions were detected at necropsy and all cultures remained sterile. Only a mild conjunctivitis limited to slight congestion and excessive lacrimation developed following reexposure. Of the 2 does rebred 6 weeks postabortion and not exposed, one delivered a normal full term litter. The other reacted as if pseudopregnant. Subsequent rebreeding of each doe resulted in normal full term litters. A few young in these 3 litters died, however, lesions could not be detected and all cultures remained sterile. These results are summarized in Table II.

Born at term. The 7 does exposed 4 days or less prepartum all gave birth to apparently normal litters. Of 58 young born, 21 were stillborn and the remainder, with one exception, expired within the first 8 postpartum days. At necropsy the newborn showed varying degrees of hepatic necrosis which ranged from pin-point foci to areas as large as 3 mm in diameter. A few livers showed marked infarction. *L. monocytogenes* was isolated from most of these young as indicated in Table I. Grossly, no other lesions were detected. Young which survived for several days usually were active and nursed until death. At necropsy the stomach often was filled with milk. One doe was sacrificed 6 days postpartum. It had delivered 9 young and *L. monocytogenes* was isolated from 6. At necropsy lesions were confined to the uterus which appeared to be undergoing normal involution. However, when this organ was opened it contained many small flecks of caseated pus and the endometrium was necrotic. *L. monocytogenes* was isolated only from the uterus. One doe died 3 weeks postpartum. Necropsy revealed an anemic uterus which contained numerous abscesses. This organ was firmly adhered to the large intestine and contained a moderate amount of cream colored pus-like fluid. There were no other lesions and *L. monocytogenes* was isolated only from the uterine abscesses. Only one of the 7 does made any attempt to prepare a nest. It was felt this reaction reflected a state of ill health. Although they did not appear acutely ill, they were listless, refused food, and failed to show the aggressiveness usually associated with kindling. Usually the does appeared asymptomatic within a few days after parturition. The only young one to survive longer than 8 days was born to a doe exposed 2 days prepartum and is presently 4 months old. It appears normal and has no demonstrable agglutinins for *L. monocytogenes*. *L. monocytogenes* was isolated from all 6 litter mates.

Rebreeding. Two does exposed 2 days prepartum, when rebred one week postpartum, aborted spontaneously during the 3rd week of gestation. One delivered only one mummified fetus and a necrotic cotyledon. The other delivered a normal appearing cotyledon and 2

TABLE II. Summary of Results.

	Total	Fate	Rebred	Reexposed	Fate	Rebred	Fate	Rebred	Fate
Exposed at end of 2nd gestation wk	13	5 aborted & survived 2 aborted & died 3 aborted & sacrificed 2 died before abortion 1 sacrificed before abortion	3 at 8 wk postabortion	At 2 wk gestation	1 aborted & died 1 died 1 normal litter*				
			2 at 6 wk postabortion	Not	1 normal litter	10 days postpartum	Normal litter		
					1 "pseudopregnant"	At expected term	Normal litter		
Exposed 72 to 96 hr prepartum	5	5 kindled. All young died 1 sacrificed 1 died 3 wk postpartum	3 at 17 days or later postpartum	Not	3 normal litters	1 at 6 wk postpartum	Normal litter		
Exposed 48 hr prepartum	2	2 kindled. All but 1 young died	2 at 7 days postpartum	Not	2 spontaneous listeric abortion at 3 wk gestation	2 at 7 days postabortion	1-8 macerated fetuses at term 1 "pseudopregnant"	5 wk postpartum At expected term	"Pseudopregnant" Normal litter
Nonpregnant	12	12, no indication of generalized infection							

* Sacrificed. Legs accidentally broken. No detectable lesions. All cultures sterile.

FIG. 2. Typical listeric conjunctivitis 9 days after instillation of *L. monocytogenes* showing edema, corneal opacity, and copious purulent exudate.

well developed 3 week old fetuses. One week later it delivered a necrotic cotyledon and 2 mummified fetuses. *L. monocytogenes* was isolated in large numbers in pure culture from all this material from both does. Each doe exhibited a marked vaginal discharge for 6 days following abortion and *L. monocytogenes* could be cultivated from the vagina of one for 8 days. These 2 does were bred again one week after abortion. One apparently was pseudopregnant and no young were born. She was rebred 4 days following anticipated term and delivered a normal appearing litter of 10. Two of these died within the 1st 3 days. There were no lesions at necropsy and all cultures remained sterile. The other delivered at least 8 completely macerated and liquefied fetuses and 4 necrotic cotyledons at term. *L. monocytogenes* was isolated in very small numbers from only one of these macerated masses. Swab cultures from the vagina failed to reveal significant bacterial growth. At no time did the doe appear ill. During the following 4 weeks all attempts to breed this doe failed as she consistently refused the buck. However, she was bred in the 5th week but no young were delivered at term. The 3 does bred 17 days or later after parturition all delivered normal full term litters. A few young which died failed to show lesions at necropsy and all cultures remained sterile. One of these does has delivered a second normal appearing litter. These results are summarized in Table II.

Nonpregnant. The characteristic eye lesion (Fig. 2) which developed in the 12 nonpregnant controls was identical to that of pregnant animals. However, throughout the course of the conjunctivitis none displayed symptoms suggestive of generalized infection. Nevertheless, 4 days after exposure *L. monocytogenes* was isolated from the vagina of 2. Two days later these 2 animals were sacrificed and *L. monocytogenes* was isolated from the uterus and vagina of one. No gross lesions in any organs including the reproductive tract were detected at necropsy. Necropsy of 6 other controls failed to reveal significant findings and all cultures remained sterile. Differential leukocyte counts were inconsistent and failed to reveal characteristic trends. A few animals showed a slight increase in the number of monocytes. Some pregnant animals which developed generalized infection displayed a moderate polymorphonuclear leukocytosis just before death. Nevertheless, in no instance could prognosis be predicted from these examinations.

Discussion. The results convincingly revealed that the conjunctivitis which developed from the instillation of *L. monocytogenes* into the conjunctival sac of the rabbit is not a local reaction as previously supposed(14-16). The female reproductive tract was found to be highly vulnerable to insult by this method of exposure, suggesting that it is one of the more susceptible organ systems to infection with *L. monocytogenes*. This emphasizes the contention of Osebold and Inouye(23) that listeriosis is actually a highly complex disease process that may occur under a wide range of manifestations.

These findings and others to be published subsequently(13) suggest that only a small number of organisms are required to initiate infection in the pregnant uterus of the rabbit. Nonpregnant animals displayed no clinical symptoms suggestive of infection indicating that only a transitory inapparent infection resulted. Agglutination studies revealed that

in general nonpregnant controls failed to develop demonstrable agglutinins whereas some does which aborted developed titers as high as 1:640. It remains to be determined whether the mere presence of embryonic tissue or hormonal factors are responsible for the rapid proliferation in the pregnant uterus. These results also confirm the findings of Levi *et al.*(24) that *L. monocytogenes* may persist for relatively long periods of time in the female reproductive tract. In the present study *L. monocytogenes* was found to survive at least 4 weeks in the reproductive tract of the rabbit.

It appeared that if the entire conceptus was expelled, the defensive mechanism of the doe could successfully combat the relatively few remaining bacteria as in the nonpregnant. However, when fragments of the infected conceptus were retained, either local abscesses containing the bacterium were formed which in some instances constituted a source of infection for the next litter, or if the retention was severe, a fatal septicemia resulted. This may explain why some does died with listeric septicemia following abortion while others survived.

It has not been determined whether the 2 does which failed to kindle following rebreeding actually were pseudopregnant, which is known to occur, or whether this failure was due to early intrauterine death resulting from the previous listeric metritis.

The conjunctivitis which developed from reexposure was not as marked as in the initial exposure. This and the fact that 2 animals died may be further confirmation of the findings of Morris and Julianelle(15) that ocular instillations may give at least a temporary local immunity, but not to the body as a whole. Any immunity which may have developed was insufficient to protect against subsequent infection. Some animals aborted in spite of an agglutinating titer of 1:1280, thus confirming the findings of Julianelle(25) that agglutinating antibodies are not a measure of protection against infection with *L. monocytogenes*.

Although conjunctivitis often is associated with listeric encephalitis of ruminants, the isolation of *L. monocytogenes* from the ruminant eye has not been reported. No mention is made of conjunctivitis among the numerous patients with listeric abortion(1-3). The reports of outbreaks of listeric conjunctivitis in the human population of the Soviet Union (26-29) make no mention of abortion. From limited data in an associated study in rabbits it appears that the presence of listeric conjunctivitis at the time of breeding has no effect on the young. However, does with conjunctivitis often refused the buck. Nevertheless, this report establishes the eye as a potential portal of entrance leading to abortion or intrauterine infection, or generalized infection in the gravid female. The lesions in the fetuses appeared to be indistinguishable from those described for the so-called *granulomatosis infantiseptica* of infants(1-3) and from those found in young foals(4,5), lambs(6,7), and calves(9-12).

The use of oblique lighting and a binocular scanning microscope(18,22) proved an invaluable aid in examining cultures prepared from grossly contaminated material. It often was necessary to culture vaginal exudate or small aborted fragments directly from the dropping pan of the cage. Neither was any attempt made to disinfect the vulva before taking vaginal cultures. In some instances the number of contaminating colonies was very high while the number of colonies of *L. monocytogenes* was very low. Nevertheless, the colonies of *L. monocytogenes* readily could be identified by their characteristic blue-green color and finely textured surface. It is doubtful that their presence could have been detected without this aid. In many other instances, in spite of what appeared to be a grossly contaminated environment, *L. monocytogenes* was recovered in pure culture. This was particularly true of isolations from cotyledons.

Summary. Abortion in rabbits consistently could be produced following ocular instillation of *Listeria monocytogenes* at the end of 2 weeks gestation, indicating that the resulting conjunctivitis is not a local reaction as previously supposed. When *L. monocytogenes* was instilled 4 days or less before termination of pregnancy, young either were stillborn or died with listeric septicemia within the 1st postnatal week. Two rabbits rebred one week

after delivering full term litters which died with listeric septicemia aborted spontaneously during the 3rd week of gestation and *L. monocytogenes* was isolated from all the aborted fetuses and membranes. Does rebred 17 days or more after abortion usually gave birth to normal full term litters. Two of 3 does re-exposed during the 2nd gestation died. Oblique lighting and a scanning microscope provided an excellent technic for identifying colonies of *L. monocytogenes* in cultures made directly from grossly contaminated material.

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Abortion, Stillbirth, Early Death of Young in Rabbits by *Listeria monocytogenes*. II. Oral Exposure.*† (21747)

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It was shown by Gray *et al.*(1) that abortion, stillbirth, and early death of young in rabbits could be induced by ocular instillation of *Listeria monocytogenes*. It is the purpose of this paper to present the results following the addition of the bacterium to the drinking water. This appeared to be a more natural avenue of entrance for the bacterium into the host. Potel(2) reported that a large percentage of the women with listeric abortion or early infant death (*granulomatosis infantiseptica*) in the Eastern Sector of Germany included raw cow or goat milk in the diet.

Material and methods. Twenty-two female and 2 male rabbits ranging in age from 8 to

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18 months were exposed to *L. monocytogenes* by adding dense suspensions of the bacterium to the drinking water. Six rabbits were exposed at the end of 2 weeks of gestation, 8 were 5 days or less prepartum, and the remaining 8 females and 2 males served as controls. Two of the pregnant does were exposed to a culture isolated by Potel(2) from the liver of an aborted infant, while the remaining 22 animals were exposed to a culture isolated from the brain of a calf which died of listeric encephalitis complicated by vit. E deficiency. Thirty-two ounce "Sani-glas" prescription bottles containing approximately 100 ml of Difco tryptose agar were inoculated with a distilled water suspension of freshly grown culture and incubated at 37°C for 18-24 hours. They were then filled with tap water, agitated to free the culture from the agar surface, and poured into a ceramic water dish. Thus each rabbit daily had access to 32 oz of a dense tap water suspension of *L. monocytogenes*. This constituted the only source of fluid intake for a period of 2 weeks. The average density of these suspensions corresponded approximately to the No. 2 tube of the McFarland Nephelometer when compared in a Cenco-Sheard-Sanford Photelometer. No attempt was made to prevent contamination of the water by other organisms. However, the water was cultured at frequent intervals and when badly contaminated a clean dish was provided. All aborted fetuses and animals which died or were sacrificed were cultured as previously described(1). All does which survived were rebred from 9 to 16 days following parturition but not reexposed.

Results. Abortion. There appeared to be no difference in the reactions of the does exposed to the 2 different cultures. Of the 6 rabbits exposed at the end of 2 weeks of gestation, 4 aborted on the 4th day and one on the 6th day after the introduction of *L. monocytogenes* to the drinking water. Two of these 5 does died of listeric septicemia within 48 hours after abortion. The most conspicuous lesion at necropsy was marked necrotic metritis. One uterus contained 10 partially resorbed fetuses and the other contained one necrotic cotyledon. Both contained a large amount of thick, yellow pus. The liver of each was mottled and yellowish in color. In one there were numerous ecchymotic hemorrhages in the middle portion of the large intestine. The kidneys were pitted and studded with petechiae. *L. monocytogenes* was isolated from the liver, spleen, uterus and vagina of each, and from the blood and milk of one. One doe which *aborted* died 5 days later. Necropsy revealed a greatly distended, dark purple, congested and necrotic uterus which contained what appeared to be large masses of caseated pus. The liver was slightly yellow. The remainder of the viscera appeared normal. *L. monocytogenes* was isolated in large numbers from the urine and uterus. Only a few colonies appeared on the liver culture and the remaining viscera failed to show bacterial growth. One doe *failed to abort* but died of listeric septicemia on the 8th postexposure day. Necropsy revealed a greatly distended, congested, and necrotic uterus which contained 9 partially mummified fetuses. These were adhered firmly to the endometrium. There was no evidence of amniotic fluid. In addition the uterus contained a large amount of caseous material which adhered to the endometrium. When this material was removed it revealed a corrugated and necrotic surface. The cotyledons were grey in color and completely necrotic. The livers of the fetuses were all very pale yellow, but due to the mummification no other lesions could be detected. *L. monocytogenes* was isolated from all the fetuses, cotyledons, and viscera of the doe.

Some aborted fetuses appeared well developed and normal for this stage of gestation. However, most appeared to have undergone extensive resorption before expulsion from the uterus. They were badly macerated and often consisted only of small fragments which were difficult to identify. Due to early development and extensive maceration it was difficult to detect gross lesions in the fetuses. Usually, however, the liver was slightly yellow and occasionally focal hepatic necrosis was observed. No other gross lesions were detected. In some instances the amniotic fluid was blood tinged. Usually the cotyledons appeared normal. The number of isolations of *L. monocytogenes* from fetuses and membranes is shown in Table I.

TABLE I. Number of Positive Cultures from Fetuses and Membranes.

	Total No.	Liver	Stomach contents	Urine	Amniotic fluid	Cotyledons
Aborted	22	14	4	—	—	1
Born at term	57	25–8*	14	4	2	17–4*

* Number contaminated and *L. monocytogenes* not observed. Figures for urine, amniotic fluid, and cotyledons represent total No. of specimens cultured.

As early as the 2nd postexposure day most does were depressed and refused food and water. Usually by the 3rd day they passed more or less of a viscid, sanguineous, mucous-like vaginal discharge which contained *L. monocytogenes* in large numbers. This persisted for several days and *L. monocytogenes* was isolated from the vagina of one doe for 10 days following abortion. Surviving does appeared asymptomatic within a week after abortion.

Rebreeding. Of the 2 does which survived, one was rebred 11 days and the other 27 days after abortion. At term each delivered a normal appearing litter. Some of these young were stillborn. Most of the remaining young died within the 1st 2 weeks of life. No lesions were seen in any of the young and all cultures remained sterile. The does displayed very little interest in the litters and death undoubtedly was due to neglect. These results are summarized in Table II.

Exposure near term. Of the 8 does exposed 5 days or less prepartum, 2 gave birth to apparently normal full term litters 30 hours after exposure. Three of the 15 young were stillborn. In the following 2 weeks during which the does were exposed to the bacterium they displayed no symptoms suggestive of illness, other than complete neglect of the young; all of which died within the 1st 4 postpartum days. Cultures from the urine and vagina of these 2 does were negative for *L. monocytogenes* throughout the 2 week period. No lesions were seen in the young at necropsy. Invariably the stomach was empty or contained only a small amount of greenish fluid. Initial cultures from the liver, stomach contents and cotyledons gave no significant growth. However, when the livers of these young and several cotyledons were macerated, refrigerated and recultured 2 months later (3), 2 livers from one litter and a cotyledon from the other revealed heavy pure cultures of *L. monocytogenes.* All other cultures were negative for the bacterium. Of the remaining 6 does, 4 kindled on the 3rd, one on the 4th, and one on the 5th postexposure day. Of 42 young born to these does, 29 were stillborn and none survived more than 5 days. The most conspicuous lesion at necropsy was varying degrees of focal hepatic necrosis. Occasionally the kidney showed petechiae. No other lesions were detected. In some instances the amniotic fluid was blood tinged. However, the cotyledons gave little if any evidence of alteration. Frequency of isolation of *L. monocytogenes* from fetuses and membranes is shown in Table I.

One doe exposed 3 days prepartum delivered 3 normal appearing young. For 3 days these young nursed and appeared to be in

TABLE II. Summary of Results.

Group	Total	Fate	Rebred	Fate
Exposed at end of 2nd gestation wk	6	2 aborted and survived 3 aborted and died 1 died before abortion	1 at 11 days and 1 at 27 days postabortion	Normal litter " "
Exposed 5 days or less prepartum	8	8 kindled. All young died within 5 days	7 at 9 to 17 days postpartum	5 normal litter 2 "pseudopregnant"
Nonpregnant				
♀	8	8, no clinical indication of illness		
♂	2	1 developed pneumonia		

excellent condition. Nevertheless, late on the 3rd day 2 young were found on their back, and showed mild convulsions and marked incoordination. The following morning all 3 young were found dead. Necropsy revealed marked hepatic necrosis and fibrinous peritonitis in 2. In the 3rd the liver was yellow, but no other lesions were detected. *L. monocytogenes* was isolated from the liver and blood of all 3, from the brain and kidney of 2, and from the stomach contents, lungs and urine of one. The only young one to survive 5 days showed definite symptoms suggestive of central nervous system involvement. In addition to incoordination and convulsions, it whined almost continually and occasionally cried out as if in great pain. In spite of these symptoms, which persisted for 24 hours preceding death, the animal nursed and at necropsy the stomach was filled with milk. The only lesion seen was focal hepatic necrosis. *L. monocytogenes* was isolated in large numbers from the liver and brain. Only one colony appeared on cultures from the lungs and urine.

Rebreeding. Seven does exposed late in gestation were rebred from 9 to 17 days postpartum. Five gave birth at term to normal appearing litters. A number of these young died soon after birth, but necropsy and culture failed to reveal significant findings. Three of the does showed normal interest in the litters while the other 2 completely abandoned the young. Two does reacted as if pseudopregnant and no young were born. These results are summarized in Table II.

Nonpregnant. The nonpregnant and male controls showed only a mild anorexia on the second and third day of exposure. With one exception they displayed no clinical indications of illness. On the 12th day one male developed difficulty in breathing which persisted for 3 days at which time it was sacrificed. Necropsy revealed only a mild pneumonia and *L. monocytogenes* was isolated from the lungs, nares, cecum, and small intestine. In spite of the isolation from the lung, the pneumonia appeared to be of different etiology as only one colony of *L. monocytogenes* was observed among a profuse and otherwise pure growth of an unidentified Gram negative rod. No lesions were detected in one other sacrificed control and all initial cultures failed to show significant bacterial growth. *L. monocytogenes* was isolated from the liver after maceration and refrigeration for 2 months.

Discussion. The results revealed that pregnant rabbits are highly susceptible to infection by orally introduced cultures of *L. monocytogenes,* whereas nonpregnant or male animals are relatively refractory to this method of exposure. The lesions in the fetuses and in the uterus of the does which died were similar to those seen in the fetuses and does following ocular instillation(1). In general, however, the lesions were far more extensive and advanced after oral exposure than after ocular instillation. In the latter route the fetuses generally were well developed and appeared quite normal for their stage of development, while after oral exposure most of the aborted fetuses showed evidence of extensive liquefaction and resorption and actually only fetal fragments were aborted. Likewise, the uterus of does which died following oral exposure showed a much more advanced necrosis than following ocular instillation. This may have resulted from a more overwhelming infective dose of the bacterium as the number of organisms was relatively much larger and duration of exposure was continued for a longer period of time during oral exposure than the single ocular exposure. As in the previous study(1) the results revealed that *L. monocytogenes* very quickly penetrated the placental barrier. It could be isolated from a cotyledon and the liver of 2 young ones born 30 hours after exposure. Further evidence of the rapid penetration of the placental barrier was found in the extensive liquefaction and fragmentation of fetuses aborted 3 or 4 days postexposure and in the marked focal hepatic necrosis seen in some young born at term.

The results of this and the previous study (1) suggest the uterine contents of the gravid animal as the primary focus of infection. In support of this, the exposed gravid animals displayed only relatively mild indications of infection provided the uterine contents were expelled quickly and completely after exposure. Adult animals which suffered a fatal

infection were those in which all or part of the uterine contents were retained. This was true whether exposure was midway or near termination of gestation. In contrast, most of the young of these does were fatally infected. Of the 115 young born to infected does at term in this and the previous study(1), only one survived. Further support for this concept is found in does rebred as early as 9 days following delivery of infected young. Most of these does delivered normal litters at term. Although some of these litters were abandoned, it indicated that the reproductive ability of the does had not been impaired by the previous infection.

That nonpregnant controls were virtually unaffected by a similar exposure suggests that hormonal differences in the gravid animal may enhance the bacterium's invasiveness and its eventual localization and rapid proliferation in the pregnant uterus and its contents. These findings reemphasize the contention that physical or physiological stress greatly augments the host's susceptibility to listeric infection.

With the exception of white mice(4) most investigators failed to infect adult animals consistently by the oral route. Murray *et al.* (5) succeeded in producing typical septicemic lesions in 3 of 6 rabbits 32 days old, but failed to infect adult rabbits by oral exposure. In an associated study in this laboratory, five 15-day-old rabbits showed no ill effect during a 2-week period in which heavy suspensions of *L. monocytogenes* were added to the drinking water. However, Osebold and Inouye(6) found that in spite of the absence of symptoms, rabbits exposed intragastrically actually experienced a low grade inapparent infection and *L. monocytogenes* was isolated from most of the viscera, but only after maceration and refrigeration of the tissue for a period of 45 days; a technic originally described by Gray *et al.*(3). This was confirmed when 2 of the 15-day-old rabbits were sacrificed after 2 weeks oral exposure to *L. monocytogenes*. Necropsy findings were insignificant and *L. monocytogenes* was isolated only from the intestine. The liver of each was macerated and refrigerated. Reculture 2 months later revealed a heavy pure culture of *L. monocytogenes* in each. Unfortunately this method was employed on only one nonpregnant control sacrificed in the present study. In retrospect it is remarkable that the pregnant or the very young animal as a subject for studies on the pathogenesis of listeriosis was neglected for so long since the original report of Murray *et al.*(5) involved rabbits that were either pregnant or less than 5 months of age. Also many investigators have emphasized the apparent, but still unconfirmed, greater susceptibility of the young of all species to listeric infections.

The 3 young in the same litter which remained asymptomatic for almost 3 days suggested that infection might have been by way of the mother's milk. That *L. monocytogenes* could be transmitted through milk was demonstrated by the postmortem isolation from the milk of one orally infected doe. Nevertheless, this could not be confirmed in an associated study in which does were orally exposed as described, beginning 7 days postpartum. Exposure was continued for 7 days. All young grew and developed normally. Four sacrificed young appeared normal at necropsy and all cultures failed to reveal *L. monocytogenes*, even after maceration and refrigeration. These results tend to confirm that infection actually was intrauterine rather than oral.

These findings also support the contention of Potel(2) that the oral route is the avenue of entrance in the widespread outbreaks of abortion and early infant death (*granulomatosis infantiseptica*) in the human population of both sectors of Germany. He found that many of the mothers included raw cow or goat milk in the diet. In one instance he isolated *L. monocytogenes* from the milk of a cow with an atypical mastitis. A woman drinking milk from this cow gave birth prematurely to twins and *L. monocytogenes* was isolated from the liver of each. The outbreak of abortion and early death of calves reported by Gray *et al.* (7) also strongly suggested oral exposure inasmuch as all dams involved were housed in the same barn and all the cases occurred within a relatively short period of time. Nevertheless, with the present knowledge it would be premature to accept this as the only portal of entry, particularly in view of the fact that

what appeared to be an identical type of intrauterine infection was produced by ocular instillation of *L. monocytogenes*(1).

A venereal mode of infection may not be overlooked in the pathogenesis of listeric abortion. Wenckebach(8) isolated *L. monocytogenes* from the urethral exudate of 5 men with gonorrhea and Gray and McWade(9) isolated the same bacterium from the cervix of a so-called repeat breeder cow. Although the microorganism appeared to have no pathogenic role in either of these instances, they do suggest the possibility of venereal transmission. Osebold and Inouye(6) reported fetal death and embryo resorption in rabbits exposed intravaginally on the 7th day of gestation. None of the does died or showed marked clinical indication of illness. Unpublished material from this laboratory revealed that rabbits exposed intravaginally during the third week of gestation may abort. However, this was not a consistent finding, but emphasizes the possibility of venereal infection.

The misconception that listeriosis is principally a disease of the central nervous system has done much to impede the unravelling of the pathogenesis of the several forms of the disease. Listeric encephalitis of ruminants, listeric septicemia and meningitis of man and monogastric animals, and abortion in all species may be quite different and distinct disease processes and each may prove to have a unique pathogenesis. Observations made of field outbreaks of listeric encephalitis in ruminants(10) suggested a method of exposure other than oral. Pallaske(11) isolated *L. monocytogenes* from the nares of 2 sheep with listeric encephalitis and Shimizu *et al.*(12) isolated the bacterium from a nasal swab of a normal sheep, thus suggesting respiratory rather than oral exposure. It is striking that listeric encephalitis of sheep and goats is most prevalent during the winter when the ewes are pregnant. Nevertheless, in a review Gray (13) found that listeric abortion in sheep has been reported but 5 times. Early death of lambs due to *L. monocytogenes* has been reported but twice, compared to at least 44 reports of encephalitis. Listeric encephalitis of goats has been reported 12 times. Listeric abortion is unreported. Listeric encephalitis in cows has been reported 30 times compared to 9 reports of abortion and 4 of early death of calves. If animals with listeric encephalitis are infected orally, it is difficult to explain, in view of the results of the present study, the low incidence of abortion in a population consisting largely of pregnant animals.

The factors which dictate the various manifestations of infection remain to be determined. It appears that there is a fundamental difference between ruminants and monogastric animals which has a role in determining the specific disease process which results from infection with *L. monocytogenes*. Gudkowa and Sacharoff(14) postulate a difference in adaptation in the individual strains which produce encephalitis and those which produce either septicemia or infectious mononucleosis and that the particular disease produced is a manifestation of the specific adaptation. They found that strains passed through brain tissue lose their ability to ferment sucrose and lactose, and the fermentation of these substances may be an index of affinity for neural tissue. It is well established that wide strain difference in ability to ferment sucrose and lactose exist, but further research is necessary to confirm this relationship. Nevertheless, the present study confirms the oral route as a possible mode of entry for *L. monocytogenes*, and that exposure by this method may cause intrauterine infection in gravid monogastric animals. It does not imply that this may be the mode of entry for all listeric infections.

Summary. When suspensions of *Listeria monocytogenes* were added to the drinking water of 6 rabbits at the end of 2 weeks of gestation, 2 aborted and survived; 3 aborted and died; and one died before abortion. When exposure occurred 5 days or less prepartum, the young suffered an intrauterine infection which resulted in either stillbirth or death within the 1st 5 days of life. Seven of 9 infected does rebred 11 to 27 days postpartum gave birth to normal litters. Nonpregnant female and male rabbits were not affected by a similar exposure.

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An Atypical Electrophoretic Peak in Serum of Patients with Familial Primary Systemic Amyloidosis. (21748)

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Familial systemic amyloidosis represents an unusual facet of the problem of primary or essential systemic amyloidosis. In our review of the literature, only 4 reports were found which emphasize the familial occurrence of the disease(1-4). The cases described by Maxwell and Kimball(1) and by Ostertag(2) presented no unusual new clinical features. Those of Andrade(3) and of Kantarjian and De Jong(4), however, broadened the diagnostic spectrum of the disease process through their reports on neurologic, ophthalmologic, and endocrine abnormalities resulting from the disease. It should be emphasized that no experimental data were included in these reports.

We have recently had the opportunity of studying in great detail, both clinically and experimentally, an unusually large family group afflicted with primary systemic amyloidosis. An extensive investigation has been carried out on this group, with emphasis on genetic implications, laboratory data including serum electrophoretic findings and lipoprotein determinations (by ultracentrifugation), skin biopsies, and bone marrow aspirations. It is the purpose of this paper to submit a preliminary report dealing with the serum electrophoretic findings on 5 members of this family.

Methods. Electrophoretic analysis of serum proteins was carried out in veronal buffer, pH 8.6, ionic strength 0.1 u. The procedure for quantitation of the protein components was essentially that of Tiselius and Kabat(5). Total protein was determined by the Biuret method(6,7). Mobility calculations were carried out according to the method described by Longsworth(8).

Results. An electrophoretic diagram representative of the findings in these 5 cases of familial primary systemic amyloidosis is reproduced in Fig. 1a. For purposes of comparison, a normal pattern from a member of this family is reproduced in Fig. 1b. The existence of an abnormal peak, associated with the globulin fraction is demonstrated. We have arbitrarily labeled this peak α_2'.

The electrophoretic analyses reported here were made on ascending protein boundaries. Satisfactory agreement was obtained, however, with descending boundaries.

The results of electrophoretic studies on these 5 patients, together with 5 normal control subjects, are summarized in Table I. Values for total protein are essentially normal in these patients. Values for albumin (per

TABLE I. Electrophoretic Data on Five Patients with Familial Primary Amyloidosis and Five Normal Control Subjects.

	Albumin		Globulin													
			Total		α_1		α_2		α_2'		β		γ			
Total protein, g/100 ml	% total protein	Mobility $\times 10^{-5}$, cm²/sec./v	% total protein	A/G ratio	% total protein	Mobility $\times 10^{-5}$, cm²/sec./v	% total protein	Mobility $\times 10^{-5}$, cm²/sec./v	% total protein	Mobility $\times 10^{-5}$, cm²/sec./v	% total protein	Mobility $\times 10^{-5}$, cm²/sec./v	% total protein	Mobility $\times 10^{-5}$, cm²/sec./v		
Normals																
6.84	63.0	–5.7	37.0	1.70	3.8	–4.7	6.9	–3.9	—	—	12.5	–2.8	13.8	–1.5		
7.03	60.9	–5.3	39.1	1.56	4.2	–4.4	7.5	–4.0	—	—	14.4	–2.9	13.0	–1.3		
7.03	65.6	–5.7	34.4	1.90	4.5	–4.9	9.7	–4.1	—	—	12.0	–2.9	8.2	–1.5		
6.84	60.4	–5.3	39.6	1.53	4.1	–4.5	8.7	–3.7	—	—	15.1	–2.6	11.7	–1.6		
7.61	61.1	–5.7	38.9	1.57	3.6	–4.8	9.8	–4.0	—	—	16.7	–2.8	8.8	–1.6		
Patients																
7.02	60.0	–5.7	40.0	1.50	4.5	–4.8	7.9	–4.1	4.8	–3.7	13.0	–2.8	9.8	–1.5		
7.17	55.3	–5.4	44.7	1.24	4.7	–4.5	4.7	–3.8	5.3	–3.3	15.7	–2.7	14.3	–1.5		
8.50	54.5	–5.7	45.5	1.20	3.3	–4.8	7.0	–4.1	5.6	–3.6	13.7	–3.0	15.9	–1.3		
7.17	57.0	–5.4	43.0	1.33	5.1	–4.6	6.6	–3.9	5.4	–3.4	14.0	–2.7	11.9	–1.5		
7.12	53.8	–5.5	46.2	1.16	3.7	–4.6	3.9	–4.1	8.4	–3.6	14.4	–2.8	15.8	–1.5		

cent of total protein) tend to be somewhat lower than normal, and values for total globulin tend to be somewhat higher, although the differences are of doubtful significance. The values for percentage of α_2 globulin tend to be lower in the patients with familial primary systemic amyloidosis than in the normal controls.

Mobility determinations carried out on the various protein fractions of these sera are also recorded in Table I. Comparing mobilities of the components of the abnormal sera with values obtained on the normal sera, it would seem that the abnormal peak lies between α_2 and β globulin.

Discussion. The 5 patients with familial primary systemic amyloidosis reported here all showed the presence of an atypical serum protein component. On the basis of mobility calculations, the abnormal peak would seem to be located between the α_2 and β globulin. This finding is substantiated by the fact that, although the values for total globulin tend to be high in these patients, the values for the normal α_2 component tend to be somewhat low. Percentages of α_1, β and γ globulins are essentially normal.

Considering the consistency of this finding in the patients with familial primary systemic amyloidosis, it is hoped that electrophoretic analysis of sera may afford a means of following the inheritance pattern and also serve as a valuable diagnostic criterion for the disease. Similarly, disappearance of the peak conceivably might offer a valuable aid for prognosis.

Further analysis of the data obtained on the relatively large number of other patients in this series is necessary. Such an analysis is presently in progress.

Summary. Electrophoretic data are presented on 5 related patients with familial primary systemic amyloidosis. The presence of an abnormal peak, located between the α_2 and β globulin fractions, is a consistent finding in these patients. We have arbitrarily called this component α_2'. The possible significance of this finding is discussed.

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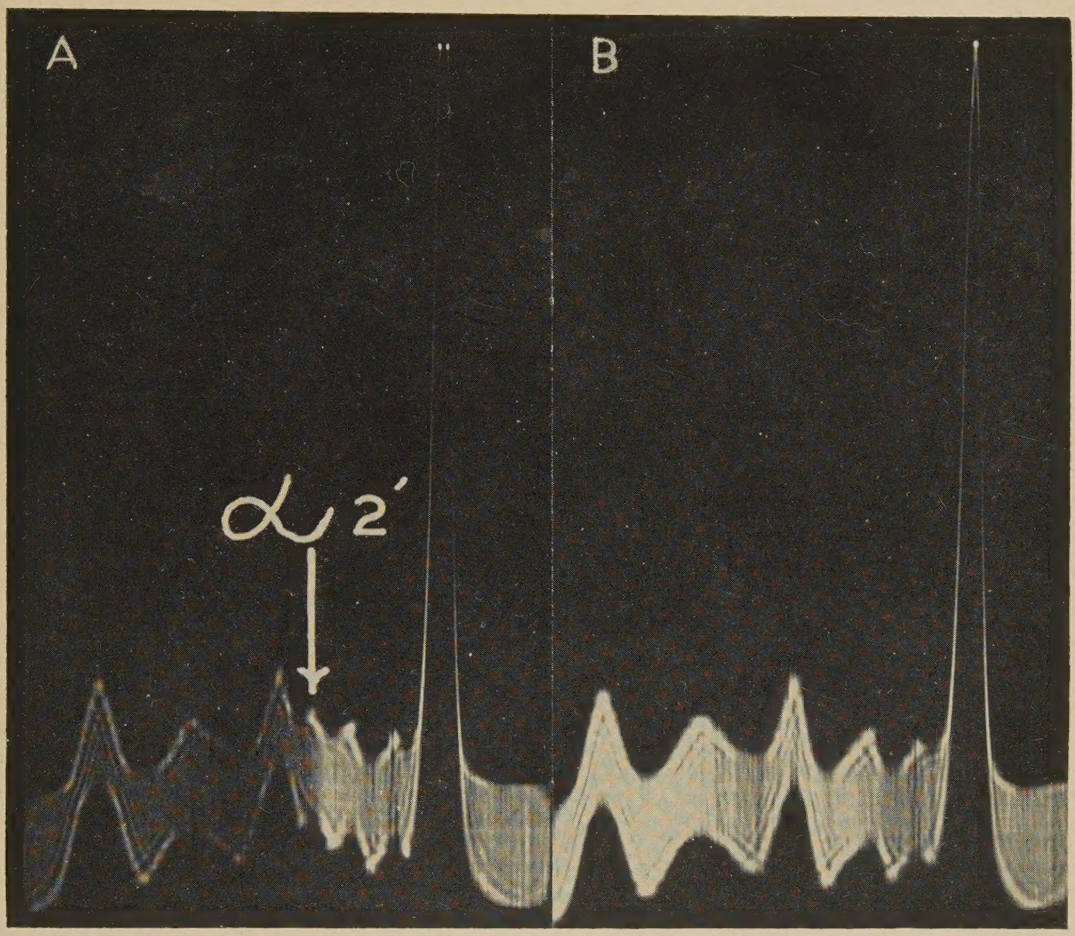

FIG. 1a. Representative electrophoresis diagram of serum in familial primary systemic amyloidosis.

FIG. 1b. Representative electrophoresis diagram of normal serum.

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Pteridines and the Nutrition of the Protozoon *Crithidia fasciculata.* (21749)

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Experiments described below led to the unexpected finding that a synergistic relationship existed between certain 2-amino-4-hydroxy-6-alkyl substituted pteridines and pteroylglutamic acid (PGA) for growth of *Crithidia fasciculata*(1). Thus the organism could be grown with a "low" catalytic level of PGA provided an appropriately substituted pteridine was also present in the medium. The experiments reported herein were part of a collaborative effort which led to the isolation from urine of a previously unrecognized pteridine, termed biopterin which was found to be active in catalytic amounts for growth of *C. fasciculata*(2). The structure of biopterin was shown to be 2-amino-4-hydroxy-6 (1′,2′-dihydroxypropyl) pteridine(2).

Methods. Microbiological procedures: The test organism, *Crithidia fasciculata,* was obtained from Dr. S. H. Hutner, Haskins Laboratories, New York City, and maintained by weekly subculture on "transfer medium." The basal medium for the assay was that described by Nathan and Cowperthwaite(3) but with the adenosine replaced by 100 γ adenine, guanine and uracil per assay tube. The organism would not grow under these conditions unless the medium was supplemented with a "high" level of folic acid, or crude natural supplements. The "transfer medium" was the basal medium plus 1 mg/ml of a crude water insoluble fraction of a papain digest of beef liver. Appropriate dilutions of test substances were added in a volume of 1 ml to a series of 11 x 180 mm test tubes, 1.25 ml of basal medium (double strength) was added to each tube and the tubes were capped, sterilized and cooled. 0.25 ml of 12% sterile glucose solution was then added aseptically per assay tube and the tubes were inoculated with one drop of a week-old culture of *C. fasciculata* which had been washed once by centrifugation and resuspension to its original volume (10 ml) in sterile saline. The tubes were incubated at 25°C for 5 to 7 days with the racks placed in a slanting position (about an 80° angle from upright) to favor aerobic conditions, following which growth was measured turbidimetrically.

TABLE I. Growth Response of *Crithidia fasciculata* to Pteroylglutamic Acid (PGA) or Natural Materials.

Additions	Folic acid of natural material at test level, mγ	*C. fasciculata* growth response, optical density
0		.07
10 mγ PGA		.09
100		.14
300		.57
1000		.88
.01 mg beef liver extract	.4	.11
.03	1.1	.89
3.8 mg brewer's yeast	84	.78
11.4	251	.98

Results. Growth Response of Crithidia fasciculata to PGA and Natural Materials: The data of Table I illustrate that a "high" level of PGA (1000 mγ/tube) supported complete growth of *C. fasciculata.* Beef liver and brewer's yeast were digested with folic acid conjugase (chicken pancreas) and the folic acid content was determined by microbiological assay with *Streptococcus faecalis*(4). These samples were then tested to see if they would promote growth of *C. fasciculata* in proportion to their folic acid content. The data of Table I illustrate that this was not the case, *e.g.* a maximum growth response was obtained to 0.03 mg of liver extract which contained only 1 mγ of folic acid.

Synergistic Effects Between PGA and Certain Natural or Synthetic Materials for Growth of C. fasciculata: Culture filtrates of *Ochromonas malhamensis,* a protozoon widely used for the microbiological assay of vit. B_{12}, were reported to support growth of *Crithidia fasciculata*(5). Several fermentations were carried out with *O. malhamensis* in which

TABLE II. Sparing Effect of Pteroylglutamic Acid (PGA) on Response of *Crithidia fasciculata* to Certain Natural and Synthetic Materials.

Additions material	Amount	*C. fasciculata* growth response (optical density) PGA omitted	10 mγ PGA added
	Exp. 1		
0		.02	.03
Ochromonas malhamensis ext.	.0004 ml	.04	.60
	.002	.26	.76
	.01	.71	.80
	Exp. 2		
Pteroylmethionine (crude)	2 γ	.04	.89
Pteroylthreonine (pure)	1	.03	.78
Pteroylaspartic acid (pure)	1	.05	.84
Pteroylglycine (crude)	.1	.03	.62
	.3	.05	.78
	1.0	.04	.90
Pteroylglycine (pure)	.1	.02	.15
	.3	.01	.42
	1.0	.04	.82

the organism was grown with vigorous aeration at 28°C in a deep tank (100 gal.) in a medium of 0.3% skim dried milk, 1.0% glucose, and 0.3% Tec Nutrient (Tec. Chem. Corp., Stamford, Conn.). In a typical experiment (Table II) 0.01 ml of an *O. malhamensis* cell extract (prepared by rupturing the cells with sand and ground glass and filtering) gave a good growth response in a medium containing no folic acid, but the remarkable finding was that in the presence of 10 mγ of PGA only about one-hundredth as much extract was required (Table II). Similar synergistic effects were found when urine collected from healthy adult males was tested with a low level of PGA(2).

A possible explanation of the synergism between natural materials and PGA for growth of *C. fasciculata* might be that the natural materials were supplying a product of the metabolism of folic acid which was not present in the basal medium. Accordingly, a number of metabolites in whose synthesis folic acid is known to participate were tested in the absence or presence of PGA including sodium formate, formaldehyde, serine, glycine, choline, thymine, thymidine, vitamin B_{12}, histidine, methionine, S-adenosyl-methionine, and a crude sample of pteroylmethionine. All of these compounds (10 γ/tube) were inactive for growth of *C. fasciculata* under both conditions except pteroylmethionine which was active in the presence of PGA (Exp. 2, Table II). Since the pteroylmethionine was not pure, some other pteroylamino acids were tested and it was found that all were active when tested with 10 mγ PGA (Table II). A finding of particular interest was that a sample of crude pteroylglycine was considerably more active for growth than the pure sample (Table II) which suggested that the crude sample might contain impurities also having growth-promoting activity.

An idea of the chemical nature of the biologically-active impurities associated with crude pteroylglycine was obtained by subjecting 2 mg of crude pteroylglycine to Craig counter-current extraction in the system n-butanol: 0.05 M pH 7 phosphate buffer, 5 ml per phase. The buffer layer was the mobile phase; after 10 transfers essentially all of the pteroylglycine was in tube 10 (as determined spectrophotometrically) whereas fluorescent material was spread throughout the tubes but was particularly strong in tube 5. This fluorescent material was absent from pure pteroylglycine. Tube 5 was concentrated *in vacuo* to dryness and dissolved in 4 ml 0.1 N NaOH. In the Beckman spectrophotometer this solution had 2 sharp peaks; a major peak at 252 mμ and a secondary peak at 360 mμ; these ultraviolet data are typical of 2-amino-4-hydroxy-6-alkyl pteridines which might be expected to be present in a crude sample of a pteroylamino acid.

Microbiological Activity of Various Pteridines: The above observations prompted the testing of a number of pteridines for growth of *C. fasciculata* in media containing traces of PGA. Microbiological activity was only observed with 2-amino-4-hydroxy-6-substituted pteridines in which the substituent (R) in the 6-position was methyl, hydroxymethyl, or formyl(2). The 6-hydroxymethyl compound at a level of .01 to .03 γ produced two-thirds maximum growth when tested in the presence of 10 mγ PGA and the corresponding level for the 6-methyl and 6-formyl pteridines was

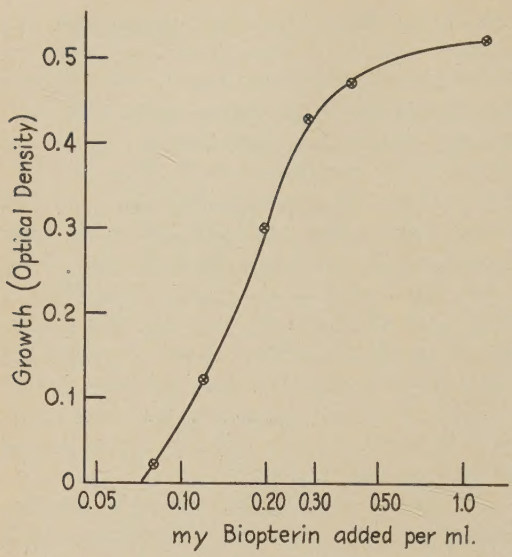

FIG. 1. Growth response of *Crithidia fasciculata* to biopterin, 2-amino-4-hydroxy-6-(1′,2′-dihydroxypropyl) pteridine.

0.1 γ. All three were inactive when PGA was omitted from the medium. The other pteridines tested were inactive at levels of 10 γ; these included 2-amino-4-hydroxy-6-substituted pteridines where R was H, COOH or OH, and also 2-amino-4-hydroxy-7-methyl pteridine, 2-amino-4-hydroxy-6, 7-dimethylpteridine and 2-amino-4-hydroxy-6, 7-dicarboxypteridine. The microbiological activity of pure pteroylamino acids (Table II) might be explained on the basis that the organism has a limited capacity to convert them to the 6-hydroxymethyl derivative or to biopterin which are utilized for growth.

Growth Response of C. fasciculata to Biopterin, 2-amino-4-hydroxy-6- (1′, 2′-dihydroxypropyl) Pteridine: The details of the isolation of biopterin from urine will be reported elsewhere. Growth of *C. fasciculata* in response to biopterin is shown in Fig. 1. The amount of biopterin required for half maximum growth of the protozoon is somewhat variable. In some experiments as little as 0.05 mγ/ml are required; in the experiment of Fig. 1, 0.2 mγ/ml of pteridine was needed. Based on the amount of urine required for growth of *C. fasciculata,* it is estimated that urine contains about 1 γ of biopterin per ml. One complicating aspect of these studies was that within the past year for unknown reasons the test organism, *C. fasciculata,* "lost" its nutritional requirement for preformed folic acid so that the growth response of *C. fasciculata* to biopterin is now obtained in the absence of added PGA. However, the involvement of PGA in the metabolism of the protozoon was demonstrated indirectly(2).

Summary. The Trypanosomid flagellate *Crithidia fasciculata* was found to have a growth requirement for certain 6-substituted pteridines. The most active compound studied was 2-amino-4-hydroxy-6 (1′, 2′-dihydroxypropyl) pteridine ("biopterin") which was isolated from human urine. A requirement for both pteroylglutamic acid and an appropriate pteridine was demonstrated for the organism.

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